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ADRENOCORTICAL INSUFFICIENCY WITH NORMAL BASAL LEVELS OF URINARY 17-HYDROXYCORTICOIDS: DIAGNOSTIC IMPLICATIONS*†

NAJIB ABU HAYDAR, M.D.,‡ JACQUES R. ST. MARC, M.D., WILLIAM J. REDDY, A.B., JOHN C. LAIDLAW, M.D.§ AND GEORGE W. THORN, M.D.

The Departments of Medicine, Harvard Medical School and the Peter Bent Brigham Hospital, Boston, Massachusetts

ABSTRACT

Adrenocortical function has been studied in 7 patients who had some of the symptoms of Addison's disease, yet had normal basal levels of 17-hydroxycorticoid excretion in the urine. Tetrahydrocortisone was found to be the major component of the urinary 17-hydroxycorticoids. Its identity was established by paper chromatographic separation and sulfuric-acid spectrum. None of the patients responded to the intravenous administration of ACTH by a rise in steroid excretion, thus demonstrating the absence of any significant adrenocortical reserve. In 6 of the 7 patients hyperpigmentation was the first symptom observed, and in 2 it was the patient's only complaint. The clinical recognition

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‡ Fellow of the Damon Runyon Memorial Fund. Present address: American University of Beirut, Beirut, Lebanon.

§ Markle Scholar in Medical Sciences. Present address: The Banting Institute, University of Toronto, 100 College Street, Toronto, Canada.

of partially compensated adrenocortical insufficiency is of practical importance, since under conditions of severe stress acute adrenal insufficiency may occur and may go unrecognized.

SINCE Thomas Addison's classic description of chronic adrenal insufficiency one hundred years ago (1), the clinical diagnosis of fully developed Addison's disease has presented little difficulty to the physician, in spite of its relative rarity. The functional concepts concerned, however, have undergone many changes as the understanding of adrenal physiology and biochemistry has progressed. In particular, the isolation of pituitary adrenocorticotropin (ACTH) and its availability in reasonably pure form have provided a specific and efficacious agent for the stimulation of the adrenal cortex. This, in conjunction with the development of practical methods for measuring adrenocortical hormones and their metabolites, has permitted a more definitive biochemical evaluation of variations in adrenocortical function. It was found that normal subjects excreted measurable amounts of urinary adrenocortical steroids, that this excretion was greatly increased by the administration of ACTH (2), and that standardized infusions of ACTH could be used to quantitate adrenocortical reserve (3, 4, 5). Patients with the manifestations of adrenocortical insufficiency excreted minimal amounts of adrenocortical steroids in the urine and did not respond to the intravenous administration of ACTH.

Recently a group of patients has been observed who showed some of the stigmata of Addison's disease, yet had normal basal levels of 17-hydroxycorticoid excretion in the urine. These patients failed to respond to the administration of ACTH by a rise in steroid excretion, thus demonstrating the absence of any significant adrenocortical reserve. A similar group of patients with detectable levels of plasma 17-hydroxycorticoids has been described by Eik-Nes *et al.* (6). The studies reported here have been presented in abstract form (7).

PATIENTS AND METHODS

Seven hospitalized patients, ranging in age from 2½ years to 70 years, were studied. 24-Hour urine collections were obtained on (a) two control days, and (b) two or more consecutive days during which the patient received 25 I.U. of ACTH intravenously in 500 ml. of 5 per cent glucose or physiologic saline over a period of eight hours, the infusion beginning between 9 and 10 A.M. In the patients who were already receiving cortisone, fluorohydrocortisone was substituted in order to reduce the amount of urinary steroid metabolites derived from exogenous sources; the average maintenance dose of fluorohydrocortisone is less than 1 mg. per day and contributes negligible amounts to urinary steroid excretion (8).

17-Hydroxycorticosteroids were determined by Reddy's modification (9) of the method of Reddy, Jenkins and Thorn. 17-Ketosteroids were measured by the method of Drekter *et al.* (10), whereby colorimetry was performed by the technic of Holtorff and Koch (11). The circulating eosinophils were counted using the technic previously de-

scribed (12). For chromatographic study, an aliquot of urine was incubated with 300 units of β-glucuronidase per ml. at pH 5 and 37° C. for forty-eight hours. It was then extracted twice with 100 volumes per cent of ethyl acetate at pH 1. The extract was washed with 1/10 volume of cold 0.1 *N* NaOH and 1/10 volume of distilled water. After drying over anhydrous Na_2SO_4, evaporation was carried out under reduced pressure at temperatures ranging between 40–50° C. The extract was fractionated on Whatman #2 filter paper in an aqueous methanol-toluene system at elevated temperature (13). The fractions were eluted with methanol and measured by the Porter-Silber reaction using cortisone as a standard.

Clinical picture. Table 1 summarizes the clinical findings in the 7 patients. Detailed histories are given in the Appendix. The youngest, M.H., manifested evidence of segmental adrenal deficiency. She was admitted to the hospital with a diagnosis of idiopathic juvenile hypoglycemia precipitated by fasting. She was capable of withstanding severe salt restriction without untoward effects. There was no evidence of female pseudohermaphrodism and the level of urinary 17-ketosteroid excretion was normal. Patient M.C. presented no subjective symptoms other than increased pigmentation. She was capable of moderate physical exertion, yet when cortisone was administered in a dosage of 25 mg. daily she felt more energetic and the pigmentation regressed appreciably. Patient M.G., in addition to pigmentation, complained of constant fatigue, a symptom which diminished during cortisone therapy. The remaining 4 patients approached the more classic picture of Addison's disease and required substitution therapy to maintain a state of well-being and normal daily activities.

TABLE 1. CLINICAL FINDINGS IN 7 PATIENTS WITH PARTIAL ADRENOCORTICAL INSUFFICIENCY

Patient	Age (yrs.)	Duration of illness before therapy (yrs.)	Pigmentation	Weakness	Loss of weight	Hypoglycemia	Water and electrolyte disturbance
M.H.	2½	1 ±	+++	0	0	++++	0
M.C.	26	6	++++	0	0	0	0
M.K.	33	¼	+++	++	++	0	++
M.G.	35	5	+++	++	0	0	0
H.K.	48	1	+++	++	0	0	++
H.W.	50	⅓	++	++++	++	0	++
H.F.	67	0	0	++++	0	++	+++

In 6 of the 7 patients, the first symptom noted was hyperpigmentation of the type seen in Addison's disease, most prominent in exposed areas, the extensor surfaces, pressure points, skin folds, scars and mucous membranes. In the seventh, adrenal insufficiency developed acutely while the patient was in the hospital receiving heparin therapy. Presumably this represented acute adrenocortical insufficiency secondary to hemorrhage into the adrenal glands. Replacement therapy was instituted promptly, perhaps preventing the compensatory mechanisms that account for pigmentation from coming into play.

RESULTS

Steroid excretion

Control. It is striking that the severity of the symptoms of adrenocortical insufficiency varied widely and could not be correlated with the levels of urinary 17-hydroxycorticosteroids, which are shown in Figure 1. The control levels of 17-hydroxycorticosteroids in all patients fell within the normal limits obtained by the Reddy method—1 to 10 mg. per twenty-four

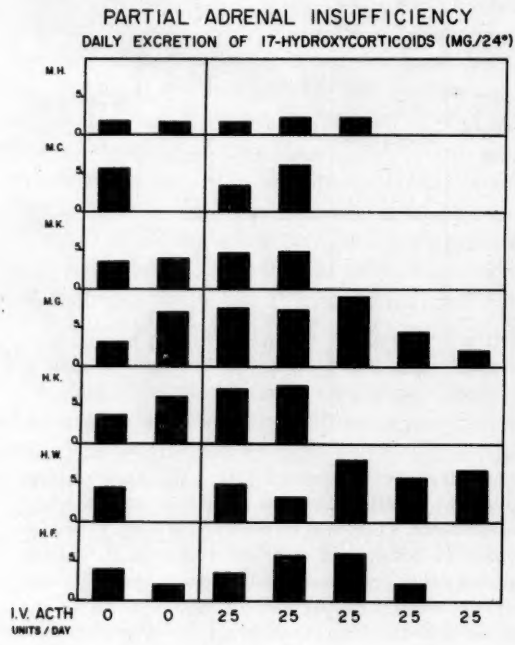

FIGURE 1

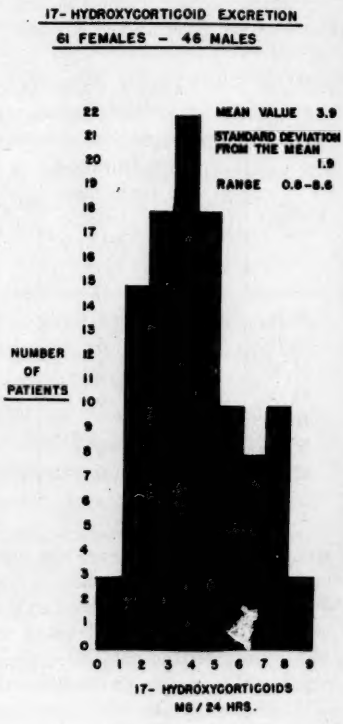
17- HYDROXYCORTICOID EXCRETION
61 FEMALES - 46 MALES
MEAN VALUE 3.9
STANDARD DEVIATION FROM THE MEAN 1.9
RANGE 0.6-8.6
NUMBER OF PATIENTS
17- HYDROXYCORTICOIDS MG / 24 HRS.

FIGURE 2

hours (Fig. 2). The day-to-day variations seen in normal subjects were also observed in the 7 patients studied. The diurnal pattern of hormone excretion (with a maximum in the morning) was checked in 2 patients (H.F. and M.K.) and found to be normal.

Response to ACTH. In all 7 patients the administration of 25 I.U. of ACTH intravenously over eight hours in glucose or saline on two or more consecutive days failed to elicit the rise in urinary 17-hydroxycorticosteroid excretion that would be expected from a normal adrenal gland (Figs. 1 and 3). However, in 3 of the patients a small initial increase in corticoid

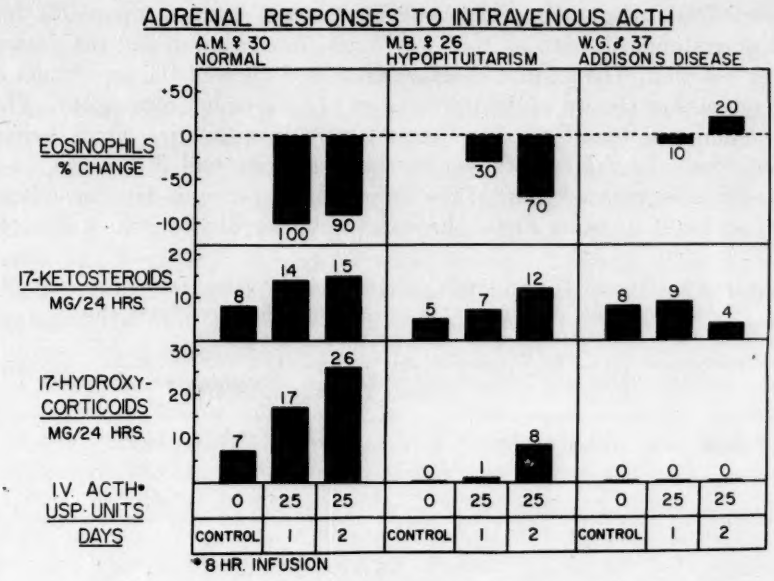

FIGURE 3

excretion was noted. This was followed by a fall in corticoid excretion to the pre-ACTH level or below on subsequent days. These changes could be interpreted as representing the discharge of stored steroids in the remaining adrenal cells or the mobilization and exhaustion of the remaining adrenal reserve. This phenomenon, though minimal, may be sufficient to explain the variation in steroid excretion from day to day and the persistence of the normal diurnal pattern of steroid excretion observed in Patients H.F. and M.K. In no instance did the rise in corticoid excretion approach that of the normal response to stimulation with ACTH. In 2 patients who were given 1 mg. of fluorohydrocortisone by mouth daily, 17-hydroxycorticosteroids disappeared completely from the urine. These observations supported the hypothesis that the chromogenic material being measured was derived from adrenocortical tissue which was under control of ACTH. Urinary 17-ketosteroid excretion paralleled 17-hydroxycorticosteroid excretion, with normal control values and failure to rise upon the administration of ACTH (Table 2). Moreover, the eosinophil count did not fall as it normally does following the administration of ACTH.

Chromatographic study

The chromatographic separation of the urinary corticoids was performed in order to ascertain the nature of the material measured by the method of

Reddy. The compounds were identified by their R_f value compared to that of a pure standard, both by the blue-tetrazolium reaction and the Porter-Silber reaction. The aliquot of urine examined allowed the separation of the major metabolite of hydrocortisone, *i.e.*, tetrahydrocortisone. This compound was found on the chromatogram in quantities which agreed closely with the values obtained by the Reddy method. Its identity was established by measurement of the sulfuric-acid spectrum. Hydrocortisone itself and 2 of its other metabolites (tetrahydrohydrocortisone and corti-

TABLE 2. URINARY 17-KETOSTEROID EXCRETION IN 7 PATIENTS WITH PARTIAL ADRENOCORTICAL INSUFFICIENCY UNDER CONTROL CONDITIONS AND FOLLOWING THE ADMINISTRATION OF ACTH

Patient	Urinary 17-KS (mg./24 hrs.)					
	Control day		ACTH test day*			
	1	2	1	2	3	4
M.H.	1.4	0.9	0.9	0.4		
M.C.	6.1	—	5.3	5.3		
M.K.	8.8	—	8.6	8.0		
M.G.	5.3	—	4.4	5.0		
H.K.	7.7	9.1	11.4	13.5		
H.W.	17.9	—	11.3	13.7	15.6	17.9
H.F.	7.9	6.5	9.5	7.8	5.1	3.9

* Dose = 25 International Units of ACTH per day, administered intravenously over an 8-hour period.

sone) were present on the chromatograms in small quantities, representing a small fraction of the total 17-hydroxycorticosteroid excretion.

Diagnosis of adrenocortical insufficiency

The final diagnosis of complete adrenocortical insufficiency rests upon the absence of adrenal steroid metabolites in the urine. Once this is established, the response to the administration of ACTH will differentiate secondary from primary adrenal insufficiency. In primary adrenal insufficiency the defect is at the adrenal level and the prolonged administration of ACTH will fail to elicit a rise in urinary corticoids. In secondary adrenal insufficiency the prolonged administration of ACTH will result in a gradual rise in the urinary corticoid level (3, 4) (Fig. 3).

The diagnosis of partial adrenocortical insufficiency is likewise dependent upon evaluation of the response to ACTH. It is the lack of increase in the urinary 17-hydroxycorticoid level after maximal stimulation with

ACTH despite normal control values which localizes the functional deficiency.

DISCUSSION

The observations presented in this study would appear to establish the existence of partial or compensated adrenocortical insufficiency. In these patients, it is at present reasonable to assume that the secretory function of the adrenal cortex was sufficient to maintain homeostasis under resting conditions, whereas evidence of insufficiency appeared under conditions of stress and increased demand for adrenocortical hormone. The degree of the insufficiency varied from one patient to another, presumably depending upon the functional reserve of the adrenocortical remnant and upon the individual needs.

Under normal conditions, the adrenal cortex is endowed with a generous functional reserve which is mobilized in conditions of stress under the effect of ACTH. The mechanism of ACTH release is still unknown, but it has been demonstrated that among the controlling factors of ACTH release are the blood level of adrenocortical hormones and, indirectly, the need of the organism for these hormones. In partial adrenocortical insufficiency, as more adrenocortical cells become nonfunctioning, the remaining cells increase their functional activity to maintain homeostasis. This compensatory hyperfunction is probably mediated by an increased secretion of ACTH. As the process of adrenocortical destruction continues, a state may be reached wherein the remaining adrenocortical cells are almost continuously working at their maximum capacity. Additional exogenous ACTH then fails to stimulate further the production of adrenocortical hormones. Under these conditions, high or maximal endogenous ACTH secretion may well be present, and this concept is fostered by the high incidence of hyperpigmentation in these patients, since it has been demonstrated that the level of melanin-stimulating hormone (MSH) secretion frequently parallels ACTH production (14–16). In 3 patients, cortisone therapy resulted in decrease of the hyperpigmentation.

That increased pigmentation may result from decreased adrenal reserve despite adequate basal secretion of adrenocortical hormones is further substantiated by observations made in Patient G.W. In this 35-year-old white male teacher, classic Cushing's syndrome gradually developed during the period 1944 to 1948. He underwent bilateral adrenalectomy in two stages in 1950. The left adrenal had to be fragmented during removal and the adrenalectomy was not considered complete on this side. Following adrenalectomy the urinary excretion of 17-hydroxycorticoids was persistently found to be within normal limits, frequently at the upper limit of normal. Stimulation with ACTH revealed definite, though decreased adrenocortical reserve (Table 3). Despite this normal baseline excretion of 17-hydroxycor-

TABLE 3. RESPONSE TO INTRAVENOUS ACTH FOLLOWING INCOMPLETE BILATERAL ADRENALECTOMY (PATIENT G.W., PBBH 8A309)

Date*	Urinary 17-hydroxycorticoids (mg./24 hrs.)				Urinary 17-ketosteroids (mg./24 hrs.)			
	ACTH (25 I.U./8 hrs.)				ACTH (25 I.U./8 hrs.)			
	Control	day 1	day 2	day 3	Control	day 1	day 2	day 3
March, 1954	5.3	14.5	15.5	18.2	9.0	10.0	14.2	13.5
March, 1955	6.8	12.4	15.5	16.7	14.3	14.9	22.4	21.2
March, 1956	8.1	16.8	16.7	16.6	9.1	8.1	15.2	12.1

* Incomplete bilateral adrenalectomy performed in 1950.

ticoids, definite and characteristic generalized pigmentation gradually developed, with accentuation at pressure points (elbows, knuckles), in the creases of the hands, and on the buccal mucosa.

The early clinical recognition of partial adrenocortical insufficiency is of practical importance, since under conditions of severe stress acute adrenal insufficiency may occur and may go unrecognized. The diagnosis of partial adrenocortical insufficiency rests on the lack of response to maximal stimulation with intravenously administered ACTH.

Acknowledgment

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APPENDIX

M.H. (*CMC 398016*), a white girl aged 2 $\frac{2}{12}$ years, had been first seen at the Children's Medical Center for treatment of bilateral, congenital dislocated hips. Subsequently she was repeatedly admitted because of episodes of loss of consciousness and convulsions associated with hypoglycemia; these episodes were precipitated by intercurrent minor illnesses associated with poor food intake. In the summer of 1954 she was admitted for study and was found to be normal for her age, weighing 27 pounds 10 ounces. The blood pressure was 115/85 mm. Hg. The skin showed generalized brownish pigmentation, with a deeper color at the sites of pressure and irritation from previous plaster casts. The liver was felt 2 cm. below the right costal margin. There was some limitation of abduction and extension in the right hip. The rest of the findings on physical examination were within normal limits. There were no genital anomalies. Laboratory findings included normal urine, normal white cell count and differential, and normal concentrations of hemoglobin, fasting blood glucose and nonprotein nitrogen. The levels of serum sodium, potassium, chloride, calcium and phosphorus were within normal limits and remained so during six days of limited sodium intake. Urinary sodium excretion decreased appreciably during the period of sodium restriction. An electrocardiogram was normal. An electroencephalogram was interpreted as abnormal because of low voltage and some fast activity in all leads. Results of an insulin tolerance test indicated increased sensitivity to insulin, as the test elicited convulsions which were relieved by intravenous glucose administration. Urinary excretion of adrenal steroids and the response

to ACTH are shown in Figure 1 and Table 2. The findings were interpreted as clearly indicating diminished adrenocortical reserve, although basal secretion apparently was sufficient for day-to-day needs in the absence of stress. It was felt that, in the presence of stress, adrenocortical function with regard to glucocorticoid activity became inadequate, whereas mineralocorticoid activity remained sufficient. Cortisone was started in a dosage of 4 mg. by mouth twice daily, and the child has done well since that time.

M.C. (*MGH 712072*), a 26-year-old single white female, was admitted to the Massachusetts General Hospital because of increasing pigmentation. There were no further complaints and, in particular, there was no history of weakness, nausea, vomiting, change in body hair or menstrual irregularity. There was no increasing fatigue and no history suggestive of morning hypoglycemia. Physical examination revealed a well developed and well nourished adult young female with deep, diffuse pigmentation of the skin, particularly marked over the exposed areas and pressure points. The areolae were darkly pigmented. Blood pressure was 102/70. The thyroid was somewhat enlarged, but no nodularity could be detected. The remainder of the physical findings were unremarkable. Laboratory data included a fasting blood glucose level of 75 mg. per 100 ml. and a normal response to intravenous insulin. Serum sodium and potassium concentrations were within normal limits and the patient's ability to handle a water load was adequate. Steroid excretion values and the ACTH response are shown in Figure 1 and Table 2. These findings were interpreted as suggesting adequate adrenocortical function for day-to-day needs but severe limitation of adrenocortical functional reserve. Administration of cortisone, 25 mg. daily by mouth, resulted in definite regression of pigmentation and an improved sense of well-being (the latter finding being purely subjective).

M.K. (*PBBH S 6495*), a 33-year-old jeweler of Jewish descent, was admitted to the Peter Bent Brigham Hospital in June 1954. Two years before admission he had noted a tumor of the anterior chest wall which had been "resected." Subsequently there had been formation of a draining fistula. After a second local surgical operation, isoniazid therapy had been started. One year before admission he noticed increasing fatigability, pigmentation, impotence, and anorexia resulting in a weight loss of 15 Kg. At that time a clinical diagnosis of Addison's disease was made. In addition, acid-fast bacilli were found in a biopsy specimen of the still-draining fistula. Therapy with desoxycorticosterone was initiated, followed by a marked increase in well-being, increased strength and appetite, and a gain in weight. On admission the patient was somewhat overweight, his blood pressure was 110/70, and he was deeply pigmented. Pigmentation of the skin overlying bony prominences was striking. There were many darkly pigmented spots on the lips and gums. Over the sixth costal cartilage on the right there was a small fistula oozing greenish pus. The liver was palpable two finger breadths below the right costal margin, and the tip of the spleen could be felt on deep inspiration. Laboratory findings included: hematocrit 48 per cent, white blood cell count 5,000 with 65 per cent lymphocytes, sedimentation rate normal, and and serum electrolyte levels within normal limits. Roentgenographic examination of the chest showed an old, retracted and probably inactive tuberculous process of the upper lobe of the right lung with deviation of the trachea to the right. The diameter of the heart was 17 per cent below the expected average, and scattered small areas of calcification were apparent in the spleen. There was no evidence of calcification in the suprarenal region on either side. An electroencephalographic tracing showed diffuse, nonspecific slowing. Urinary excretion of steroids and the response to ACTH are shown in Figure 1 and Table 2. A therapeutic regimen was started which included 50 mg. of desoxycorticosterone trimethylacetate by intramuscular injection

once monthly, 12.5 mg. of cortisone acetate by mouth daily, 150 mg. of isoniazid daily and 12 Gm. of para-aminosalicylic acid daily. On this program the patient continued to improve, gained weight, and the impotence disappeared. He was readmitted in September 1954, at which time a partial resection of the 5th and 6th right costal cartilages, including the fistulous tract, was performed. During anesthesia and the immediate postoperative period the patient was given hydrocortisone intravenously, then additional cortisone by mouth. Preoperative maintenance levels of hormonal replacement therapy were reached within five days. He has since been maintained on 0.5 mg. of fluorohydrocortisone by mouth daily and is asymptomatic. The pigmentation is still marked.

M.G. (*PBBH 4G695*), a 35-year-old white female, was admitted in October 1954 because of increasing pigmentation. Her past history was unremarkable until five years before admission, when she first noted the appearance of dark freckles which increased progressively in size, then merged into diffuse, generalized pigmentation. Otherwise, she had felt perfectly well. Two years before admission a further increase in pigmentation became apparent, particularly in pressure areas, and she observed a definite darkening of her nipples and the appearance of pigmented spots on her gums. She continued to feel well, with the exception of a mild degree of weakness and fatigability, but without anorexia, diarrhea, weight loss or changes in axillary or pubic hair. One year before admission she was given 25 mg. of cortisone acetate daily without effect. On physical examination the patient was a well developed, well nourished young female with generalized pigmentation as described in the history. There were several areas of pigmentation on the gums. The skin was rather fine and smooth; the distribution and growth of body hair was normal. There was no thyroid enlargement, and no lymphadenopathy. The blood pressure was 110/85. The remainder of the examination revealed no other abnormalities. Laboratory findings included: hematocrit 41 per cent, white blood cell count 7,900 with 47 per cent lymphocytes, concentration of fasting blood glucose 77 mg. per 100 ml., blood urea nitrogen 9 mg. per 100 ml., serum total protein 5.7 Gm. per 100 ml. with albumen 4.4 Gm., and serum cholesterol 228 mg. per 100 ml.; serum bilirubin concentration and thymol turbidity were within normal limits. Thyroidal I^{131} uptake was 46 per cent at twenty-four hours, the basal metabolic rate was −10 per cent, and an electrocardiographic tracing was within normal limits. X-ray examination of the thorax and abdomen revealed a cardiac diameter 15 per cent below the expected average, and scattered discrete calcifications in the upper lobe of the right lung, interpreted as probably old, inactive tuberculosis. No calcification was seen in the region of the adrenals. The urinary excretion of steroids and the ACTH response are shown in Figure 1 and Table 2. It was felt that the patient's adrenal reserve was adequate for daily routine living but inadequate for situations involving severe stress, and presumably inadequate to suppress pituitary secretion of melanophore-stimulating hormone. A therapeutic program was started which included 37.5 mg. of cortisone daily and 50 mg. of desoxycorticosterone trimethylacetate by intramuscular injection once monthly. Considerable decrease of pigmentation was noted during the following months.

H.K. (*PBBH A 9067*), a 48-year-old white man, was first admitted in November 1954. From 1932 to 1936 he had been hospitalized in the Rutland State Sanatorium for treatment of extensive pulmonary tuberculosis. He was then followed at regular intervals, and there had been no evidence of recurrence until May 1953, when he first noted the onset of increasing weakness and fatigability along with sexual impotence. By March 1954 these symptoms had become severe. In addition, generalized pigmentation was commented upon by his family and he complained of anorexia, occasional vomiting, and abdominal cramps. His temperature was elevated to 101° F. and he was admitted to the

Rutland State Sanatorium where he was found to be febrile, dehydrated, and pigmented; his blood pressure was 80/60, hematocrit 50 per cent, blood urea nitrogen level 87 mg. per 100 ml., and serum potassium level 6.8 mEq. per liter. There was no roentgenologic or bacteriologic evidence of recurrent pulmonary tuberculosis and an intravenous pyelogram showed good excretion of the dye without evidence of renal pathology. Culture of the urine for acid-fast bacilli yielded negative results. The presence of Addison's disease was suspected and replacement therapy was initiated (cortisone 12.5 to 37.5 mg. per day and desoxycorticosterone trimethylacetate 30 mg. once monthly) along with chemotherapy for tuberculosis (streptomycin, 1 Gm. daily for eight days followed by 1 Gm. twice weekly for four months; isoniazid, 200 mg. daily). He was referred to the Peter Bent Brigham Hospital for evaluation. On admission, he was found to be in good general condition, with a blood pressure of 130/74 and normal temperature and pulse. There was definite generalized pigmentation, particularly marked over the dorsum of both hands, over the elbows and knees, and in the palmar creases. The perineum and scrotum were darkly pigmented and there were several small pigmented spots on the mucous membrane of the gums and lips. Axillary and pubic hair was normal. The pulmonary findings were consistent with the interpretation of fibrosis of the right lung secondary to pulmonary tuberculosis. No further anomalies were noted. Laboratory findings included hematocrit 45 per cent, normal white blood cell count and differential, and normal sedimentation rate; the level of fasting blood glucose was 64 mg. per 100 ml., serum sodium 140 mEq. per liter, and potassium 3.2 mEq. per liter. In the electrocardiogram there was some flattening of the T waves. X-ray examination of the chest showed no evidence of activity of the fibrotic process in the right lung. No acid-fast bacilli were found in the sputum or in material obtained by gastric aspiration. The steroid excretion levels and ACTH response are shown in Figure 1 and Table 2. The patient has since been maintained on 50 mg. of desoxycorticosterone trimethylacetate intramuscularly once monthly and 6.2–12.5 mg. of cortisone acetate daily by mouth. He now feels well and leads a normal, active life; sexual impotence has disappeared; there has been some decrease in overall pigmentation.

H.W. (*PBBH 5 H20*), a 50-year-old white male, was first admitted in December 1954 because of increased fatigability and weight loss. In 1928 pleural effusion had been noted, and in 1942 pulmonary tuberculosis localized to the right apex was recognized and treated with rest for one year. In 1950 reactivation of the tuberculous process occurred, with cavitation of the right apex. This was treated by resection of the right upper lobe and the major portion of the right middle lobe, and by right-sided thoracoplasty. Following these procedures stabilization appeared to have been reached, although during the summer of 1954 a fistula to the left anterior part of the thorax developed. Chemotherapy with dihydrostreptomycin and isoniazid was initiated. On admission the patient's blood pressure varied between 100/60 and 130/80; temperature and pulse rate were normal. In addition to the pulmonary findings, minimal diffuse pigmentation of the skin and many darkly pigmented freckles were noted. There was no unusual lymphadenopathy, and the remainder of the physical examination failed to reveal further anomalies. Laboratory findings included: hematocrit 42 per cent, normal urine, level of fasting blood glucose 63 mg. per 100 ml., serum sodium 124 mEq. per liter (later 137 mEq.), serum potassium 5.0 mEq. per liter (later 3.5 mEq.), and serum chloride 88 mEq. per liter (later 99 mEq.). Roentgenographic examination of the lungs revealed no evidence of activity of the pulmonary disease and no acid-fast bacilli were found by smear or culture of material obtained by gastric aspiration and of washings from the fistula tract. The size of the heart was below the predicted average; electrocardiographic and electroencephalographic tracings were normal. Steroid excretion and ACTH response are shown in Figure 1 and

Table 2. During admission to the hospital chemotherapy was continued and the patient was given, in addition, 0.5 mg. daily of fluorohydrocortisone. This resulted in complete return to normal of the serum electrolyte levels, and in restoration of subjective well-being. Six weeks later, at another hospital, resection of a cold abscess and part of the 10th rib on the left side was performed. Hydrocortisone was administered intravenously during and immediately following the operation. The postoperative course was uneventful. The patient has been followed at intervals since, and has remained in excellent condition. There has been little change in the pigmentation.

H.F. (*PBBH 9 B 874*), a 67-year-old salesman, was first admitted to the surgical service of the Peter Bent Brigham Hospital in 1952 because of right-sided intermittent claudication. His blood pressure was 180/85. A right lumbar sympathectomy was carried out and postoperative treatment included anticoagulation therapy with heparin. A large hematoma in the region of the surgical incision was noted about one week following operation and three days later the patient became progressively lethargic and hypotensive; the level of blood glucose was found to be 43 mg. per 100 ml., and of serum sodium, 123 mEq. per liter. Dramatic improvement followed the administration of glucose and desoxycorticosterone, and on discharge the patient was told to take desoxycorticosterone and cortisone. The intermittent claudication was markedly improved. He was readmitted six weeks later for evaluation of adrenal function. A diagnosis of adrenocortical insufficiency, possibly secondary to adrenal hemorrhage, was established on the basis of the absence of a decrease in circulating eosinophils or an increase in urinary 17-ketosteroids in response to intravenous ACTH stimulation over eight hours on two consecutive days. The patient was then maintained on 25 mg. of cortisone acetate by mouth daily and was regularly followed in the metabolic clinic. When readmitted in January 1955, he was a spry elderly man in apparent good health. His blood pressure was 200/90. There was no evidence of increased pigmentation. There were small tophi at the edge of both pinnae, and ophthalmoscopic examination revealed moderate narrowing and tortuosity of the arterioles. The thyroid was not enlarged. The heart was slightly enlarged to the left and a harsh, grade II apical systolic murmur was heard. There was a left direct inguinal hernia. The testes were normal in size and consistency. No arterial pulsations were felt below the knees but both lower extremities were warm—the right somewhat warmer than the left. Laboratory findings included normal urine, normal hematocrit, and white blood cell count and differential. The concentration of blood glucose was 80 mg. per 100 ml., blood urea nitrogen 14 mg. per 100 ml., serum sodium 135 mEq. per liter, potassium 4.9 mEq. per liter, uric acid 6.6 mg. per 100 ml., and cholesterol 215 mg. per 100 ml. Thyroid indices were normal. Roentgenographic examination of the chest revealed a heart diameter 18 per cent above the predicted average (versus normal heart size in 1952) and many small calcified areas in both lungs suggesting healed histoplasmosis. These calcifications had already been noted in 1952 and there had been no change. The steroid excretion and ACTH response are shown in Figure 1 and Table 2. The left inguinal hernia was repaired during this admission and the postoperative course was uneventful. The patient is now taking 12.5 mg. of cortisone daily and continues in satisfactory condition.

TREATMENT OF CUSHING'S SYNDROME WITH AMPHENONE: REPORT OF TWO CASES, ONE WITH PROBABLE THYMOMA

E. PERRY McCULLAGH, M.D. AND
HARVEY A. TRETBAR, M.D.

The Department of Endocrinology, The Cleveland Clinic Foundation, and The Frank E. Bunts Educational Institute, Cleveland, Ohio

ABSTRACT

Two patients with Cushing's syndrome were treated with amphenone before undergoing adrenal surgery. One of these patients also had an anterior superior mediastinal neoplasm of probable thymic origin. The other suffered from severe mental depression, and manifested unusual adrenal sensitivity to corticotropin. The administration of amphenone in each case resulted in decreased formation and excretion of glucocorticoids, as shown by changes in the levels of 17-hydroxycorticoids in the plasma and urine, as well as other metabolic and biochemical effects secondary to these changes. In Case 1, during intravenous infusion of amphenone, urinary 17-ketosteroids were unchanged; during oral therapy they were depressed less than the corticoids. In Case 2, during oral therapy, urinary 17-ketosteroids were depressed, but more slowly than the corticoids.

AMPHENONE, synthesized by Allen and Corwin in 1950 (1), was selected by Hertz and associates (2, 3) for biologic study because of its progesterone-like effects. In the course of their studies amphenone was found to cause enlargement of the adrenal glands and, to a lesser degree, of the thyroid. Further inquiry showed that these changes did not occur in the hypophysectomized animal, and that concomitant administration of cortisone prevented adrenal enlargement without interfering with the goitrogenic effect (4). Likewise thyroid feeding prevented thyroid hypertrophy without modifying the adrenal enlargement. It was presumed that hypertrophy both of the adrenals and of the thyroid following amphenone feeding was due to inhibition of the production of the respective hormones, leading to an increased secretion of thyrotropin and corticotropin. Hume and Nelson (5) in experimental studies on acutely hypophysectomized dogs found that the administration of corticotropin caused active production of adrenocortical steroids, which could be markedly depressed by giving amphenone. This finding was confirmed by Tullner, Graff and Hertz (6). *In vitro*, adrenal tissue slices when provided with suitable precursors are similarly inhibited in the formation of 17-hydroxycorticoids (7). Rosenfeld and Bascom (8) utilizing adrenal-perfusion methods in the

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calf showed that amphenone interferes with the synthesis of hydrocortisone, cortisone, and corticosterone.

Irregular reduction of both plasma and urinary 17-hydroxycorticoid levels was observed when amphenone was administered to patients who had extensive metastatic cancer of the breast, and at necropsy hypertrophy of the adrenal cortex was discovered (9). Thorn *et al.* (10), and Hertz *et al.* (11, 12) observed greatly decreased secretion of carbohydrate-active corticoids following amphenone treatment of patients with spontaneous or induced hyperadrenocorticism. This was shown by decreases in the levels of plasma and urinary 17-hydroxycorticoids, blood sugar concentration, glycosuria, and insulin requirement. The excretion of 17-ketosteroids was not uniformly affected. Later studies (13) suggested that the variability of these findings may have been due to a colored urinary metabolite of amphenone, and that this could be eliminated by pretreatment of the urine with exchange resins.

Liddle *et al.* (14) and Renold *et al.* (15, 16) have suggested that amphenone also diminishes urinary aldosterone in a variety of clinical syndromes, and does so occasionally in dosages that have no effect on 17-hydroxycorticoid excretion.

Peterson, Hertz and Lubs (17), utilizing isotope dilution methods, recently established that in man the action of amphenone is the direct suppression of the biosynthesis of adrenal steroids.

The occasional association of Cushing's syndrome with neoplasms of thymic, bronchial and pancreatic origin is well documented (18–39). The occurrence of such tumors in association with adrenal hypercorticism is significant because of, 1) the frequent development of these neoplasms in patients at a much younger age than usual, 2) the unusually severe metabolic derangements, and 3) the particularly fulminant course of the associated hypercorticism.

We were confronted with a case of severe Cushing's syndrome associated with an anterior mediastinal cancer thought to be of thymic origin. Because of the severity of the illness, it seemed desirable to evaluate the effects of amphenone in an attempt to improve the patient's clinical status. Similarly, the effects of amphenone were evaluated in another patient with severe psychic manifestations associated with Cushing's syndrome.

METHODS

Amphenone (1,2-bis (*p*-aminophenyl)-2-methylpropanone-1-dihydrochloride) was given intravenously in 5 per cent dextrose in distilled water at a rate of approximately 1.0 Gm. per hour. Close observation was maintained of the effects on pulse, respiration and blood pressure. Orally, amphenone capsules containing 0.5 Gm. were given at intervals varying from one and one-half to four hours. Several times, early morning doses were omitted. Both patients remained in the hospital throughout the evaluation period.

Urinary excretion of 17-hydroxycorticoids was determined by the method of Silber and Porter (40), the normal range in adults being 2–10 mg. per twenty-four hours. Urinary excretion of 17-ketosteroids was estimated by the method of Callow *et al.* (41), the normal range in adults being 4–18 mg. per twenty-four hours.

The plasma 17-hydroxycorticoid concentration was determined by the Peterson *et al.* (42) modification of the Silber-Porter method (40), the normal range being 5–20 μg. per 100 ml. Response to exogenous corticotropin (ACTH) was evaluated by administering 25 mg. of ACTH in 5 per cent dextrose and distilled water intravenously over a period of six to eight hours. The control values for 17-ketosteroids and 17-hydroxycorticoids were estimated on a 24-hour specimen of urine collected the preceding day and compared to the values found during the twenty-four hours subsequent to the time the ACTH infusion was begun.

Aldosterone activity was determined by the bioassay method of Liddle (14), which is based on the electrolyte excretion of adrenalectomized dogs that are maintained on a fixed dosage of cortisone.

Oral glucose tolerance tests were performed using 100 Gm. of glucose in a single dose

CASE 1

A 39-year-old man was admitted to the hospital on March 27, 1956, with the diagnosis of Cushing's syndrome and an associated anterior mediastinal neoplasm considered to be thymic or bronchogenic carcinoma. He had been well until the spring of 1954 when he noted a 15-pound gain in weight, swelling of the neck and shoulders, loss of libido, and hoarseness. At a hospital elsewhere, biopsy of a lymph node in the neck showed "undifferentiated carcinoma," and a roentgenogram of the chest revealed what seemed to be an anterior mediastinal tumor. He was treated elsewhere by conventional x-ray therapy in June 1954, and in July 1954 he received 6000 r of cobalt^{60}. He worked part of the following year, but in October 1955 he noted the onset of weakness that was progressive, and in January 1956 the diagnosis of Cushing's syndrome was made.

The past history was noncontributory, his only surgical treatment having been orchidopexy in 1943 for an undescended testis. There was no familial history of endocrinopathy, cancer or diabetes.

Physical examination revealed a weak, chronically ill man with a round, plethoric face, obesity about the neck and shoulders, thin arms and legs, and moderate hoarseness. The skin was parchment thin and there was a pustular acneform eruption over the arms, shoulders and trunk, with cellulitis over the left anterior chest and right thigh. Depilation and tanning of the skin about the neck and the upper and anterior chest from previous x-ray therapy was present. The blood pressure varied from 170 to 220 mm. Hg systolic and 100 to 130 mm. Hg diastolic, the pulse was 140 per minute, weight 190 pounds, and temperature 99° F. Funduscopic examination revealed only mild constriction of the arterioles. There was pitting edema (2 plus) of the feet. Slight fullness was evident in the right upper abdomen, but no definite mass was palpable. The right testis was atrophic. There was weakness of the extremities, most marked in the legs, as well as a slight tremor of the hands.

Laboratory studies on admission showed the urine to be clear, with one-plus sugar, a trace of albumin, pH 6.0, and no acetonuria. The hemoglobin concentration was 11.0 Gm. per 100 ml., the hematocrit 38 per cent, and the red blood count 4.28 million per cu.mm. with 6.2 per cent reticulocytes. The leukocyte count was 20,500 per cu. mm. with 91 per cent neutrophils, 4 per cent lymphocytes, 3 per cent monocytes and 2 per cent myelocytes. Platelets appeared to be normal. The fasting blood sugar level was 213 mg.

per 100 ml. Blood urea concentration was 33 mg. per 100 ml., and the CO_2-combining power 36.7 mEq. per liter. Serum sodium concentration was 147 mEq., potassium 3.5 mEq., and chloride 99 mEq. per liter. The results of blood Wassermann and Kahn tests were negative. The serum calcium level was 9.9 mg., phosphorus 3.0 mg., total protein 5.3 Gm., albumin 3.5 Gm., and globulin 1.8 Gm. per 100 ml. The 5-OH indoleacetic acid excretion was 1.9 mg. per twenty-four hours. The 24-hour urinary excretion of 17-ketosteroids was 30.0 mg. and of 17-hydroxycorticoids, 24.6 mg. *Micrococcus pyogenes albus*, coagulase positive, was cultured from the regions of cellulitis. Following sensitivity studies, appropriate antibiotic therapy was initiated.

Indirect laryngoscopy revealed paralysis of the right vocal cord. X-ray examination of the chest showed considerable widening of the superior mediastinum, which had a transverse diameter of 8.5 cm. In the lateral view, the anterior portion of the superior mediastinum appeared to be occupied by this mass, but there was no encroachment on the lumen of the trachea. Examination of the dorsolumbar spine revealed slight generalized demineralization. A urogram showed an indefinite shadow above the left kidney, which was suggestive of an enlarged adrenal gland. Findings from presacral air insufflation were interpreted as demonstrating bilaterally enlarged adrenal glands.

Review of the slides of the lymph-gland biopsy specimen received from another institution revealed a malignant epithelial neoplasm compatible with bronchogenic or thymic carcinoma. The location of the apparently primary site of the neoplasm associated with the duration and apparent response to radiation therapy favored thymic etiology (Fig. 1).

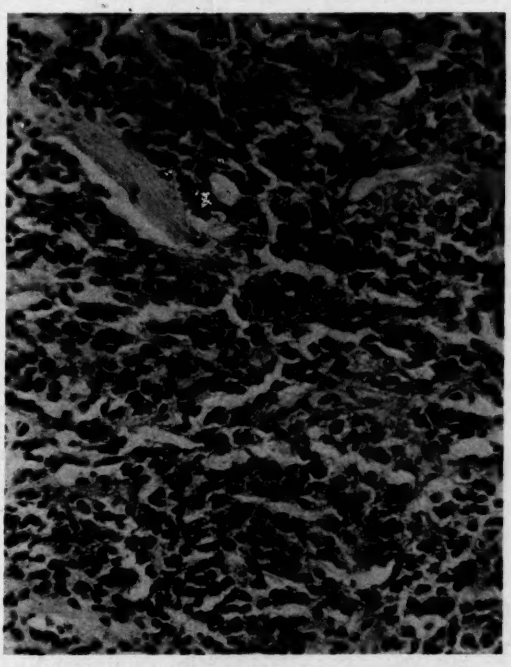

FIG. 1. (Case 1.) Photomicrograph of biopsy specimen of cervical lymph node. Hematoxylin-eosin stain; ×270.

Treatment. In spite of treatment with oral potassium, the patient's weakness became even more marked. The serum potassium level was 2.9 mEq. per liter, and an electrocardiogram revealed the characteristic changes of hypokaliemia. These changes were reversed by the intravenous administration of 80 mEq. of potassium chloride in addition to the 60 mEq. he received by mouth daily. The serum potassium concentration rose to 3.7 mEq. per liter ten hours after the infusion.

A daily dosage of 45 units of NPH and 10 units of regular insulin was required to maintain near-normal blood sugar levels until after amphenone was started. The psychic state was one of marked apprehension, but the patient remained coherent and oriented until a day prior to his death.

On his tenth hospital day, he was given amphenone intravenously, 1.0 Gm. per hour for five hours. Determinations of plasma and urinary corticoids, 17-ketosteroids and aldosterone as well as other laboratory studies were made at intervals. At 8:00 A.M. the following day insulin was omitted and oral amphenone was administered. He received this drug at intervals of one and one-half to three hours for six days. No further insulin was given.

Variable degrees of mild abdominal distention developed, and the patient became quite drowsy—though easily aroused. Occasional twitching of the muscles was noted but no abnormal reflexes were observed. He had difficulty in raising pharyngeal and bronchial secretions and complained of dyspnea, for which intermittent aspiration was performed and oxygen was given by nasal catheter. Prostigmine on several occasions provided some relief of the abdominal distention but had no effect on the profound weakness.

Throughout the period of administration of amphenone blood pressure values remained nearly normal until the fourth day of treatment, when they became variable. On the seventh day, amphenone therapy was stopped at 8:30 A.M. At 3 P.M., *bilateral total adrenalectomy* was performed by Dr. E. F. Poutasse. The patient's systolic blood pressure varied between 100 and 180 mm. Hg during this procedure and at its conclusion laryngeal edema developed, requiring a tracheotomy. About two hours after the adrenalectomy was concluded he died suddenly while receiving hydrocortisone and norepinephrine intravenously. He had received approximately 250 mg. of hydrocortisone by vein in the preceding three hours. Resuscitation was unsuccessful. Permission for necropsy was not granted.

Pathologic findings. After dissection of fat, the left adrenal measured 8.5×3.8×1.4 cm. and weighed 23 Gm. The right adrenal measured 7.2×3.7×1.1 cm. and weighed 17 Gm. (Normal adrenal weights are 5 Gm. to 7 Gm. per gland.) The medulla was glistening gray-white, and scant. The external surface of the glands was smooth with rounded margins, and the color was mottled golden-yellow and brown. Sections revealed thickened non-nodular cortices with the golden yellow of the parenchyma predominating. Cortical width was 0.5 cm. Microscopically there was a marked increase of the zona fasciculata and reticularis, and some increase in cytoplasm with excessive lipoid vacuolization. The glomerulosa seemed to be unaffected. No evidence of any cytotoxic effect was observed. There was mild focal plasma-cell and lymphocytic interstitial infiltration.

Results

The acute intravenous administration of amphenone produced dramatic metabolic and chemical effects (Figs. 2 and 3, and Table 1). The blood pressure decreased, and plasma corticoid values fell rapidly and markedly. There was a striking depression in urinary corticoids while 17-ketosteroids

remained unchanged. The patient became somewhat drowsy during the administration of amphenone but at no time did the blood pressure or general condition suggest hypocorticism. The serum potassium level rose from 3.7 mEq. to 5.6 mEq. per liter immediately following the five-hour infusion of amphenone and dropped to 3.5 mEq. eight hours later. There was no striking change in the values of serum sodium, chloride, or carbon dioxide,

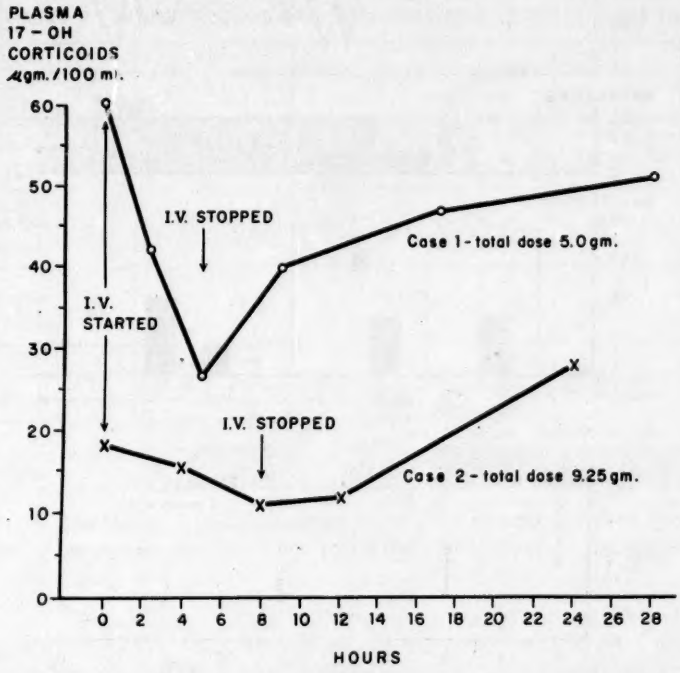

FIG. 2. Effect of intravenous amphenone on levels of plasma 17-hydroxycorticoids. (Cases 1 and 2.)

which were determined before, and at four-hour intervals after administration of amphenone.

During oral amphenone therapy, the blood sugar level fell and insulin no longer was necessary with the daily dietary intake remaining at approximately 2,000 calories. Urinary assays[1] revealed only traces of aldosterone (less than 6 μg. per twenty-four hours) prior to, and during the first, third and fourth days of treatment. Within seventy-two hours after oral amphenone was begun, the blood pressure became variable, tended to rise, and one-plus to two-plus "glycosuria" developed despite normal blood

[1] We are greatly indebted to Dr. Grant Liddle for the performance of these bioassays.

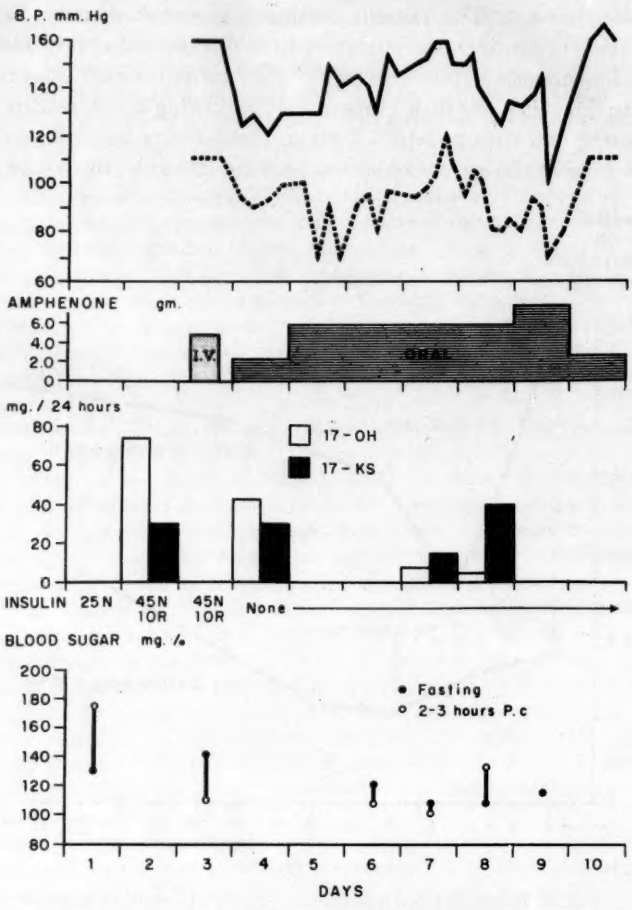

FIG. 3. (Case 1.) The effect of amphenone on blood pressure, urinary 17-hydroxycorticoids and 17-ketosteroids, fasting and post-cibum blood sugar levels, and insulin requirement.

sugar values. On the fifth day of oral therapy (day 8, Fig. 3) the patient was given 0.5 Gm. amphenone at 3 A.M. and at 6 A.M.; at 9 A.M. the dosage was increased to 0.5 Gm. every one and one-half hours. This change resulted in a fall of blood pressure; however, the positive results with urine reduction tests persisted, possibly because of an excretory metabolite of amphenone, as described subsequently by Hertz *et al.* (11) and Lanthier *et al.* (13). As may be seen in Figure 3, the fasting blood sugar values on days 4, 5 and 6 of oral administration of amphenone were 100, 100 and 108 mg. per 100 ml. respectively.

During prolonged administration of amphenone the urinary 17-hydroxycorticoids remained depressed. The urinary 17-ketosteroid levels dropped to 15.7 mg. per twenty-four hours on the third day of oral therapy and increased to 42.6 mg. on the fourth day. The CO_2-combining power of the blood fell to 21.4 mEq. per liter on the fourth and fifth days.

TABLE 1*

EFFECTS OF INTRAVENOUS AND ORAL AMPHENONE ON ELECTROLYTES AND CO_2

	4/3	4/6 F	4/6 5 hrs.	4/6 9 hrs.	4/7 17 hrs.	4/8	4/9	4/10	4/11
Sodium (mEq./L.)	146.5	137.5	142.5	142.5	146.5	142.5	140	148	146.5
Potassium (mEq./L.)	2.9	3.7	5.65	5.3	3.5	3.95	4.05	4.15	3.8
Chlorides (mEq./L.)	96	100	100	100	100	104	104	105	104
CO_2 (mEq./L.)	32.9	29.5	29.1	28.7	28.7	28.7	26.9	21.4	21.4
Amphenone		I.V.			ORAL				

* Case 1. The change in blood chemical values prior to intravenous amphenone administration was apparently due to oral and intravenous potassium therapy (see text).

CASE 2

A 41-year-old white woman, whose condition had been diagnosed as Cushing's syndrome, was first seen October 5, 1956. Previously she had had 5 pregnancies, with 4 normal deliveries and 1 miscarriage. She had been well until thirty months previously, when she consulted her local physician because of acne over the chest and back, a rapid 20-pound gain in weight, hirsutism, absent libido, weakness of the legs, and the development of a round, red face. At that time hypertension was first noted. After a six-month episode of hypermenorrhea, amenorrhea ensued. Her physician reported that in February 1955 the urinary 17-ketosteroids measured 15 mg. per twenty-four hours and that following intravenous ACTH this value increased to 35 mg. She was believed to have Cushing's syndrome, and she was given a course of x-ray irradiation to the pituitary gland, which was concluded on December 2, 1955. Thereafter she noted temporary improvement and return of menses; however, with subsequent exacerbation, weakness, agitation and depression occurred. Tranquilizing and antihypertensive agents were of little help. She stated that there was no polyuria or polydipsia and no familial history of diabetes or endocrinopathies.

Physical examination revealed an apathetic, depressed woman with obesity of the trunk, a cervicodorsal hump and acne vulgaris of the upper torso. Xanthelasmata were present and the abdomen showed purple striae laterally. Her height was 65½ inches,

weight 183½ pounds, blood pressure 180/120 mm. Hg, and pulse rate 92 beats per minute. Upon funduscopic examination, minimal arteriolar attenuation and sclerosis could be seen. The rest of the findings were not remarkable.

Laboratory studies showed the urine to be clear with a pH of 5.5. The hemoglobin level was 11.0 Gm. per 100 ml., the hematocrit 36 per cent, and the leukocyte count 10,700 with 81 per cent neutrophils, 2 per cent eosinophils, 10 per cent lymphocytes and 7 per cent monocytes. Results of an oral glucose tolerance test were as follows: fasting blood sugar level, 94 mg.; half hour, 154 mg.; one hour, 141 mg.; two hours, 138 mg.; three hours, 62 mg.; and four hours, 48 mg. per 100 ml. There was no glycosuria. The serum sodium level was 148 mEq., potassium 4.2 mEq. and CO_2 combining power 24.8 mEq. per liter. The blood urea concentration was 30 mg. per 100 ml., and urea clearance was 80 per cent the first hour and 83 per cent the second hour. The concentration of serum cholesterol was 358 mg., and of serum calcium 9.6 mg. per 100 ml. Results of blood Wassermann and Kahn tests were negative. The urinary excretion of 17-ketosteroids was 10.8 mg., and the 17-hydroxycorticoids 15 mg. per twenty-four hours. Following administration of 25 mg. of ACTH intravenously on two occasions, the 24-hour excretion of corticoids rose to 53 mg. and 73 mg., and the 17-ketosteroids to 15.5 and 25 mg., respectively.

X-ray examination of the chest revealed healed fractures of the seventh and eighth ribs. Films of the vertebrae showed wedging and collapse of the third dorsal vertebra and moderate generalized demineralization of the lumbosacral spine. Films of the skull demonstrated a normal sella turcica. The right parietal bone showed a 1-cm. cleanly punched-out radiolucency which was thought to resemble an eosinophilic granuloma. An intravenous urogram was not remarkable. A presacral air insufflation study was not entirely satisfactory, but was thought to suggest an enlarged right adrenal gland.

Treatment. On October 15, 1956, a total of 9.25 Gm. of amphenone was given intravenously over an eight-hour period at variable rates necessary to decrease blood pressure (Fig. 4). The rates of administration of amphenone varied from 0.5 to 2.0 Gm. per hour, with a short latent period between the increased rate of administration and the effect on blood pressure. During the infusion the patient became drowsy and relaxed, though exhibiting a clear sensorium and less depression than immediately before treatment. On one occasion the following day she experienced mild vomiting without associated nausea. She also felt "light-headed," but did not become depressed until approximately twenty hours after the infusion.

Seven days later, 0.5 Gm. of amphenone was given by mouth at four-hour intervals and continued for three and a half days. After approximately twelve to eighteen hours of therapy her psyche again improved. The effects on blood pressure were less impressive than following intravenous administration. On October 29, 1956, *right total and left subtotal adrenalectomies* were performed by Dr. E. F. Poutasse. A total of 21 Gm. of tissue was resected, leaving an estimated 2–3 Gm. *in situ*. During the first seventy-two hours postoperatively both intramuscular and intravenous hydrocortisone were required for control of hypotension and weakness.

Her psyche was improved for six days postoperatively; then depression and agitation with increasing suicidal tendencies were noted. Rapid progression of her agitated depression led our consultants in neuropsychiatry to recommend electroconvulsive therapy despite the increased risk. Precautionary measures were taken, which included abdominal binding, additional intravenous hydrocortisone before and after treatments, diacetylcholine chloride (anectine chloride), and thiopental sodium. Postoperatively, moderate hypertension persisted despite decreased doses of exogenous corticoids. The daily dosage of hydrocortisone was decreased to 120 mg. on the sixth postoperative day and to 40

mg. on the eighth postoperative day. She was discharged on the fortieth postoperative day, taking 10 mg. of hydrocortisone daily. She received further electroconvulsive therapy as an out-patient. A roentgenogram of the skull on March 25, 1957, showed no change in radiolucency. A sternal marrow aspiration was performed, but results of microscopic examination were nondiagnostic.

Pathologic findings. The right adrenal measured 8×3 cm. and weighed 11.65 Gm. It was smooth except for a few 1-cm. nodules at one edge. Cortical width was 1–2 mm. The left adrenal (major portion) measured 6.5×2×2 cm. and weighed 9.65 Gm., though the medulla was absent. Microscopically there was a thickened cortex and an apparent reduction in medullary tissue. The cortical cells were increased in size in the zona fasciculata and reticularis, but were present in normal numbers (75 cells in thickness). The zona glomerulosa showed no marked change. There was an over-all increase in cortical elements in some regions, with extracapsular nodular accumulation. Abundant lipid was observed, as well as vacuolization. There were focal collections of lymphocytes in the deep cortex.

Results

The effects of acute intravenous administration and of prolonged oral administration of amphenone to this patient produced results similar to those in Case 1, though not so striking (Fig. 2, 4, 5 and Table 2). The glucocorticoids were decreased, as measured by plasma and urinary 17-hydroxycorticoids, and the blood pressure was depressed. The urinary 17-ketosteroids at first were higher than before treatment, but in three days' time they also were depressed. The apparent improvement of psyche on the two occasions of amphenone administration is interesting. It roughly paralleled the decrease in glucocorticoids. However, this may have been more apparent than real, and related to the sedative effect of the drug

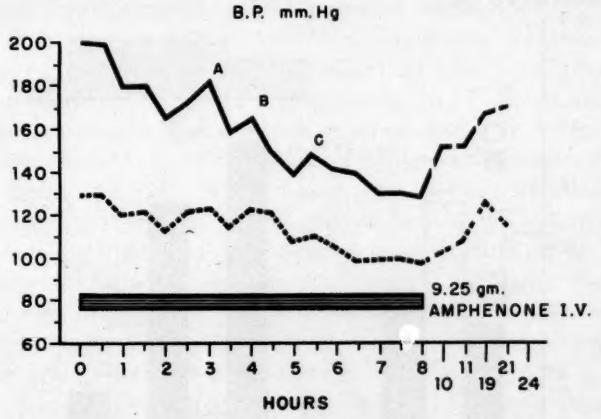

FIG. 4. (Case 2.) Effect of intravenous amphenone on blood pressure. Apparent "escape" phenomena were reversed at points A, B and C by increasing the rate of infusion.

(43). During the intravenous administration of amphenone, the serum sodium level decreased significantly, and the serum potassium level decreased slightly, though control values of the latter were normal at the onset. Urinary 17-ketosteroids again were increased after administration of amphenone by either route. At no time did a clinical syndrome of hypocorticism develop while the patient was receiving amphenone. Drowsiness and mild gastro-intestinal signs were the only observed side-effects.

DISCUSSION

The data presented demonstrate that the secretion of glucocorticoids by hyperplastic adrenal cortices was inhibited by the administration of amphenone. This was shown by: 1) decreased plasma corticoid levels, 2) de-

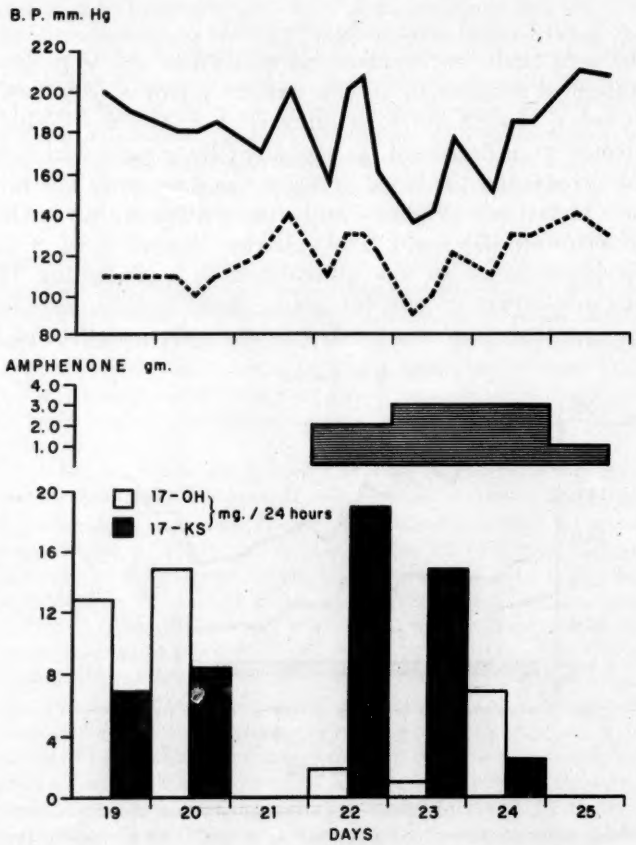

FIG. 5. (Case 2.) Effect of oral amphenone on blood pressure and urinary excretion of 17-hydroxycorticoids and 17-ketosteroids.

TABLE 2*

EFFECTS OF INTRAVENOUS AMPHENONE ON ELECTROLYTES AND BLOOD COUNT

	10/12	10/15 F	10/15 4 hrs.	10/15 8 hrs.	10/15 12 hrs.	10/16 24 hrs.
Sodium (mEq./L.)	148	146	140	132.5	140	140
Potassium (mEq./L.)	4.2	4.0	4.05	3.75	3.9	4.4
Chlorides (mEq./L.)	100	109	109	105	108	108
WBC	10700	9900	7550	9800	7700	9100
Neutrophils	81%	71%	87%	88%	84%	79%
Lymphocytes	10%	27%	13%	12%	16%	18%
Hemoglobin (Gram %)	10.5	10.5	11.3	9.9	10.2	11.1
Cell Volume	35%	35%	36%	33%	33%	37%
Amphenone						

* Case 2.

creased urinary corticoid excretion, 3) decreased blood pressure, 4) decreased blood sugar levels, and 5) elimination of the requirement for insulin, which had been 45–55 units daily in Case 1. The blood pressure response usually was proportional to the dose of amphenone. Thus, it seems established that, in man, administration of amphenone decreases the glucocorticoid output of hyperplastic adrenocortical tissue, whether or not it is ACTH-dependent. The effect of amphenone on 17-ketosteroid excretion was somewhat variable. The technical problem involved in the determination of 17-ketosteroids in the presence of urinary amphenone metabolites has been mentioned (11). Nevertheless, the findings indicate that amphenone may act at a point in the biosynthesis of 17-hydroxycorticoids which is not in the direct path of the compounds yielding the major portion of 17-ketosteroids found in the urine (9). However, Gallagher *et al.* (44, 45), employing techniques that preclude such artifactual effects, reported marked suppression of dehydroisoandrosterone excretion in a case of adrenal carcinoma treated with amphenone.

In the reports concerning amphenone therapy, little attention has been called to its effect on blood pressure. The intravenous infusion of 9.25 Gm. of the substance in Case 2 decreased blood pressure from 200/130 to 130/96 mm. Hg. An apparent tendency to "escape" was rapidly counteracted by increasing the rate of infusion. This effect may be valuable as a

diagnostic tool, since a number of patients with hypertension and apparently normal adrenocortical function have not reacted similarly (46). However, the direct depressant action of the drug on the central nervous system (43) may play a role in the hypotensive effect.

On no occasion during treatment with amphenone did a clinical state suggestive of hypocorticism develop, as reported by Thorn *et al.* (10). This was thought to occur on one occasion while their patient was receiving 8 mg. of 9-alpha-fluorohydrocortisone per day. Hertz, Pittman and Graff (9) reported a somewhat similar occurrence in a case of adrenal carcinoma which was associated with significant methemoglobinemia.

No serious side-effects were observed in our patients, who received up to 9.25 Gm. of amphenone per twenty-four hours. Moderate abdominal distention and drowsiness occurred in Case 1, and drowsiness and vomiting on one occasion in Case 2. There was no evidence of any cutaneous abnormality due to the drug, or of clinically apparent methemoglobinemia, bone-marrow depression, or thyroid enlargement.

The fact that aldosterone excretion was low initially and remained low following treatment with amphenone must be interpreted with reservation, since only one control determination was obtained.

The extreme degree of adrenal hyperplasia in Case 1 was probably induced to a considerable extent by the administration of amphenone, but the amount of enlargement due to the drug as compared to that due to Cushing's syndrome cannot be estimated.

Acknowledgment

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ESTIMATION OF GROWTH HORMONE CONTENT IN A SINGLE HUMAN PITUITARY

CARL A. GEMZELL, M.D.* AND CHOH HAO LI, PH.D.

The Hormone Research Laboratory and the Department of Biochemistry, University of California, Berkeley, California

ABSTRACT

Growth hormone was purified from single human pituitaries by means of the chromatographic procedure previously used for pooled pituitaries, and the hormonal content of the single glands was estimated by zone electrophoresis on starch and by biologic assay in hypophysectomized rats. The amount of growth hormone in a single human pituitary was found to be between 2.7 and 5.6 mg. When the same procedure was used on single pituitaries from cattle and sheep and on groups of 10 monkey pituitaries, the amounts were found to be comparable. There was more variation from gland to gland among the human pituitaries than among glands from any other species, indicating that certain variable physiologic factors that are involved in the case of the human patients from whom the glands were obtained may have to be taken into consideration in future studies of this sort. The results of this study demonstrate that enough hormone can be recovered from a single gland for qualitative and quantitative studies.

GROWTH hormone has recently been isolated from human pituitaries collected from autopsy cases (1) and from patients undergoing hypophysectomy (2).

In the study reported in this paper the chromatographic procedure used previously for pooled pituitaries (3) was applied to the purification of growth hormone from a single human pituitary. In each case, the material obtained from a single gland was analyzed both qualitatively and quantitatively for hormonal content by zone electrophoresis on starch, and by biologic assay in hypophysectomized rats.

MATERIAL AND METHODS

The human pituitaries were obtained at autopsy, one to three days after death, in an unselected group of patients. The whole pituitary was immediately frozen, treated with cold acetone, and lyophilized. Since it was thought that manipulation of the gland before it was frozen might destroy the hormonal activity, no attempt was made to separate the anterior lobe from the posterior lobe.

The dried pituitary was cut into small pieces with a scissors and then ground. Extraction was performed in a cold CaO solution at pH 10.3 for four hours, with stirring. About

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* Tora Wåhlin Fellow from Svenska Läkarsällskapet (Stockholm). Present address: Karolinska Sjukhuset, Stockholm 60, Sweden.

10 ml. of solution was used per 100 mg. of dried gland. After centrifugation of the extract, the clear supernatant fluid was slowly brought to half saturation with respect to $(NH_4)_2SO_4$ by the addition of an equal volume of saturated $(NH_4)_2SO_4$ solution. The solution was allowed to stand overnight in a cold room (4° C.). The precipitate (Fraction B) that formed was centrifuged off, dissolved in a phosphate buffer of pH 5.1 containing 0.45 *M* $(NH_4)_2SO_4$, and dialyzed against the same buffer for at least twelve hours. The clear extract was submitted to chromatography on Amberlite IRC-50 (XE-97) resin under the conditions described previously (2). The material in the active peak was collected, dialyzed for at least forty-eight hours, and lyophilized. The final product was designated Fraction D.

Zone electrophoresis on starch was carried out by a modification of the method of Kunkel and Slater (4) as employed in this laboratory (5, 6). The starch was packed into small semicylindrical glass troughs measuring 1.5×0.7×40 cm. A 2-mg. portion of Fraction D was dissolved in 0.1 ml. of veronal buffer (0.01 *M* hydrogen veronal, 0.04 *M* sodium veronal, and 0.06 *M* NaCl) of pH 8.3, and placed on the trough. After the run, the starch in the trough was cut into 1-cm. segments, each of which was eluted with 3 ml. of distilled water. All runs were performed in a cold room at 3° C., with a potential difference of about 200 volts across the ends of the trough. Primate growth hormones were allowed to run for twenty-four hours, and bovine and ovine hormones for forty-eight hours. The protein content in the eluate was estimated by the method of Lowry *et al.* (7) on aliquots of 1 ml. The protein eluted from the segment representing the active peak was dialyzed, lyophilized, and weighed. The final product was designated Fraction Z.

The biologic potency of Fraction D was determined by the tibia test in hypophysectomized rats (8). A highly purified preparation of human growth hormone was used as a reference standard.

RESULTS

Growth hormone was purified from a 1.98-Gm., acetone-dried sample from 10 pooled human pituitaries (Table 1). The yield of the fraction designated D (tubes 104–123) was 136.0 mg. (Fig. 1), or 6.9 per cent. When Fraction D was submitted to zone electrophoresis on starch, the active material migrated away from the origin as the main component (Fig. 2).

TABLE 1. PURIFICATION OF POOLED ACETONE-DRIED HUMAN PITUITARIES

Fraction	Weight		Bioassay		
	Mg.	%	No. of rats	Dose per animal (μg.)	Average tibial width (μ)
Acetone powder	1980.0	—	5	1000	267 ±3.1*
Fraction B	275.1	13.8	—	—	—
Fraction D	136.0	6.9	4	40	221 ±2.4
Fraction Z	76.0	3.8	5	40	255 ±2.2
Fraction Z_1†	5.8	0.3	5	40	202 ±6.2

* Mean ±S.E.

† Fast-moving component (see Fig. 2).

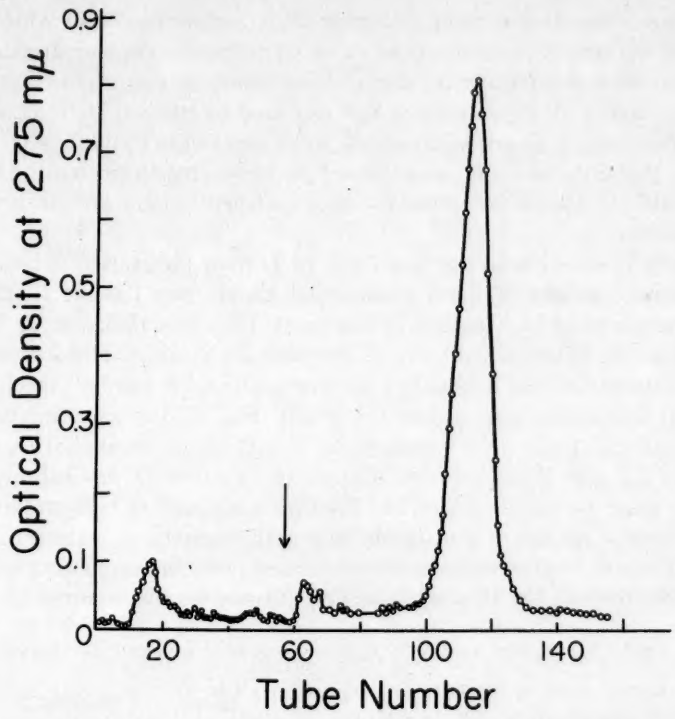

FIG. 1. Chromatography on the Na form of Amberlite XE-97 resin (dimension of column, 2.5×28 cm.) of growth hormone obtained from pooled human pituitaries; 15 ml. per tube. The hormonal activity is located in tubes 104–123. The arrow marks the change to the pH 6.0 phosphate buffer containing 0.35 *M* Na^+, 0.45 *M* $(NH_4)_2SO_4$.

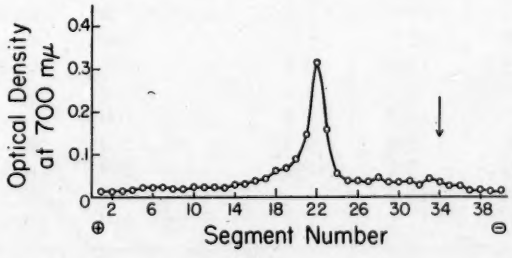

FIG. 2. Zone electrophoresis of pooled human growth hormone on starch in veronal buffer at pH 8.3. The hormonal activity is located in segments 21–23. The arrow marks the origin of the material

There was some fast-moving material (Z_1) (segments 17–20) which consisted of less than 5 per cent of the material originally applied. In addition, there was some material that remained stationary at the origin. The active peak (segments 21–23), Fraction Z, was found to contain 76.0 mg. of protein (3.8 per cent). Fractions D and Z, when assayed in hypophysectomized rats by the tibia test (8), were found to have growth-promoting effects comparable to that of a standard highly purified human growth hormone preparation.

Growth hormone was purified from 11 human pituitaries individually. The average weight of the acetone-dried glands was 193 mg. (Table 2). The average yield of Fraction D (tubes 81–100) was 15.5 mg. or 8.1 per cent (Fig. 3). When an aliquot of Fraction D obtained from these individual pituitaries was submitted to zone electrophoresis on starch, some material remained stationary at the origin (Fig. 4). It was estimated that in each pituitary the active component (Fraction Z) amounted to, on the average, 2.7 mg. When another aliquot of Fraction D was submitted to biologic assay by the tibia test, the fraction was found to be approximately 37 per cent as potent as the standard growth hormone preparation.

The amount of growth hormone recovered from the single ox and sheep pituitaries or from the 10 pooled monkey pituitaries was comparable to the

TABLE 2. GROWTH HORMONE CONTENT OF SINGLE HUMAN PITUITARIES

No. of pit.	Wt. of single pit. (mg.)	Wt. of Fraction D (mg.)	Wt. of Fraction Z (mg.)	Bioassay of Fraction D			
				No. of rats	Dose per animal (μg.)	Average tibial width (μ)	GH content (mg./pit.)
H33	221	33.7	8.1	5	40	209 ±4.7*	15.2
H35	220	44.7	5.0	4	40	198 ±1.4	14.9
H401	194	15.4	5.0	4	40	226 ±3.4	15.4
H402	199	12.3	0	5	100	189 ±1.5	0
H403	211	11.0	0.8	4	200	211 ±3.2	1.2
H404	202	8.0	0.9	4	100	212 ±3.3	1.6
H405	176	10.6	2.6	4	100	202 ±3.6	1.4
H408	176	2.0	0.2	5	200	240 ±1.9	0.7
H409	176	8.0	0.4	4	200	214 ±4.9	0.9
H410	191	11.3	5.1	4	100	237 ±3.6	6.7
H411	161	13.0	2.0	5	100	229 ±3.0	5.5
n	11	11	11				11
Mean	193	15.5	2.7				5.8
S.D.	19.6	12.5	2.7				6.0

* Mean ±S.E.

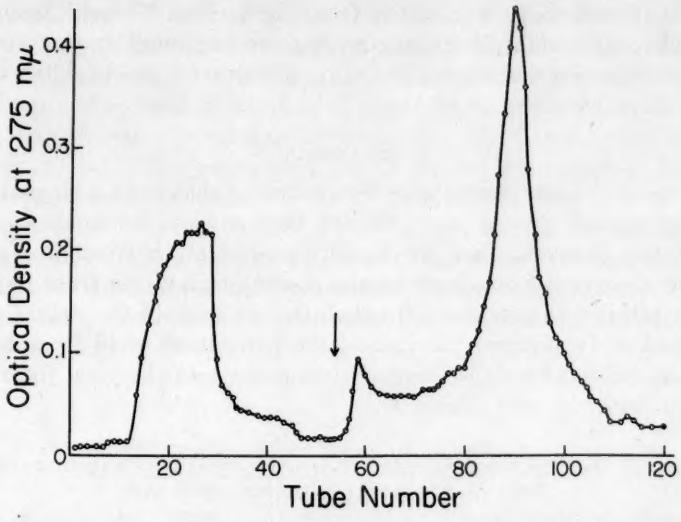

FIG. 3. Chromatography of growth hormone from a single human pituitary (H411, Table 2). The hormonal activity is located in tubes 81–100. Dimension of column 1.4×14 cm.; 3 ml. per tube. The arrow marks the change to the pH 6.0 phosphate buffer.

amount obtained from the human pituitaries. It is apparent from these data that there was more variation in the yield of growth hormone from pituitary to pituitary in the case of the human glands than there was among glands within any of the other species.

The electrophoretic mobilities of the purified growth hormone preparations from the various species were found to differ when they were submitted to zone electrophoresis on starch in a veronal buffer under identical conditions. The two primate hormones migrated a distance of from two to three times that of the bovine hormone, whereas the ovine hormone did

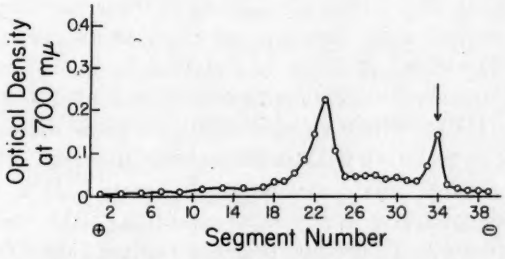

FIG. 4. Zone electrophoresis of a single human pituitary (H401) on starch. The hormonal activity is located in segments 22–24. The arrow marks the origin of the material.

not differ significantly in mobility from the bovine. These differences in electrophoretic mobility between the primate hormones on the one hand and the ovine and bovine hormones on the other indicate differences in isoelectric points (2).

DISCUSSION

The results of this study have demonstrated that from a single human pituitary enough growth hormone can be recovered for qualitative and quantitative determination. By the same procedure, the amount of growth hormone recovered from single ox and sheep pituitaries or from 10 pooled monkey pituitaries was also estimated. On the basis of the weight of acetone-dried or lyophilized pituitaries, the percentage yield from a single human gland was found to compare favorably with the yield from single glands of other species (Table 3).

TABLE 3. GROWTH HORMONE CONTENT OF THE PITUITARY IN MAN COMPARED WITH THAT IN MONKEYS, CATTLE AND SHEEP

Source	No. of pit.	Avge. wt. per pit. (mg.)	Avge. wt. of Fraction D		Avge. wt. of Fraction Z		Average GH content per pit.	
			mg.	%	mg.	%	mg.	%
Man	11	193	15.5	8.1	2.7	1.4	5.7	2.9
Monkeys	30	9.4	0.7	7.5	0.14	1.5	0.25	2.7
Cattle	3	345	25.7	7.5	5.2	1.5	6.9	2.0
Sheep	4	147	14.7	10.0	3.7	2.5	5.2	3.5

The average yield of Fraction D from a single human pituitary was 15.5 mg., with a spread of from 2.0 to 44.7 mg. When this fraction was submitted to studies by zone electrophoresis, and to biologic assay, it was apparent that it was contaminated with a large percentage of inactive proteins; it was also observed that the amount of these contaminants in Fraction D varied considerably from one pituitary to another (Table 2). One pituitary (H402) yielded 12.3 mg. of Fraction D, but when this material was submitted to zone electrophoresis no active component was obtained. Another gland (H401) which yielded about the same amount of Fraction D manifested a large active peak in zone electrophoresis. A third pituitary (H35) yielded almost three times as much Fraction D as did H401, but both D fractions gave rise to the same quantity of the active component in zone electrophoresis. Consequently, it is evident that Fraction D is not pure enough to serve for estimation of the content of growth hormone in the pituitary.

Fraction Z can be used to provide quantitative information about the content of growth hormone in a single pituitary. When samples of Fraction D from the same species were submitted to zone electrophoresis on starch under identical conditions, the active zone was consistently found in approximately the same location on the starch trough. However, the amount of protein found in the active peak was in general less than was expected from the results of the biologic assay of Fraction D. This may be due to contaminants in Fraction D which may operate synergistically with the growth hormone. The actual level of growth hormone in the human pituitary may therefore be somewhere between the estimate obtained by zone electrophoresis, as a minimum value, and that obtained by biologic assay as a maximum value.

The yield of growth hormone from a single human pituitary varied con-

TABLE 4. AMOUNTS OF VARIOUS HORMONES IN THE HUMAN PITUITARY

Hormone	Age	Amount (mg.)	Reference	Comment
Corticotropin (ACTH)	Fetus, 16 wks.	traces	Taylor *et al.* (9)	
	Fetus, 21–27 wks.	0.0005–0.001	Taylor *et al.* (9)	1 mg. = 100 U
	Infant	0.01–0.14	Taylor *et al.* (9)	
	Adult	0.20–0.24	Burns *et al.* (10)	
	Adult	0.17–0.63	Taylor *et al.* (9)	
	Adult	0.15	Hewitt *et al.* (11)	
Thyrotropin (TSH)		0.15	Chance *et al.* (12)	1 mg. = 20 U
		0.1	Currie *et al.* (13)	
		0.25–0.5	Bates *et al.* (14)	
Somatotropin (growth hormone)		3–6	Gemzell *et al.* (1)	
		2.7–5.6	present investigations	
Lactogenic hormone (prolactin)		3	Chance *et al.* (12)	1 mg. = 30 U
Follicle-stimulating hormone (FSH)	Infant	0	Bahn *et al.* (15)	
	Child	6 units	Bahn *et al.* (15)	1 mg. = ? U
	Adult, male	100 units	Bahn *et al.* (15)	
	Adult, female	200 units	Bahn *et al.* (15)	
	Menopausal female	600 units	Bahn *et al.* (15)	
Interstitial-cell stimulating hormone (ICSH)	Infant	0	Bahn *et al.* (15)	
	Child	6 units	Bahn *et al.* (15)	1 mg. = ? U
	Adult, male	100 units	Bahn *et al.* (15)	
	adult, female	200 units	Bahn *et al.* (15)	
	Menopausal female	600 units	Bahn *et al.* (15)	

siderably from gland to gland, whereas the yields from single ox and sheep glands were more consistent. This may be due to the fact that the human pituitaries were obtained from autopsy cases at varying lengths of time after death and were probably subjected to different handling after removal. On the other hand, the ox, sheep, and monkey glands were taken from healthy animals that were in the same age range, and that were sacrificed in a similar way; in addition, their pituitaries were excised promptly after death, and were all handled in the same way. It should be emphasized that the physiologic factors, such as age and sex of the patients and the nature of the final illness, may well have exercised some influence on the content of the hormone in the human pituitaries.

Table 4 summarizes all the data that have been reported in the literature concerning the amounts of known anterior pituitary hormones present in the human gland. It is of interest that the content of growth hormone seems to be high in comparison with that of other hormones. However, these data are based on biologic assays and are therefore difficult to compare on a weight basis.

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PEPTIDE-LINKED IODOTYROSINES AND IODOTHYRONINES IN THE BLOOD OF A PATIENT WITH CONGENITAL GOITER*

LESLIE J. DEGROOT, M.D., SHOLEM POSTEL, M.D., JORGE LITVAK, M.D. AND JOHN B. STANBURY, M.D.

with the assistance of FLORENCE A. CHESNUT, B.A.

The Department of Medicine, Harvard Medical School and the Medical Services of the Massachusetts General Hospital, Boston, Massachusetts

ABSTRACT

A case of congenital goitrous hypothyroidism is reported. Studies were made to determine whether a metabolic defect was present. Intravenously administered I^{131}-labeled diiodotyrosine and thyroxine were metabolized normally. Electrophoresis of plasma proteins after *in vitro* addition of I^{131}-labeled thyroxine demonstrated normal amounts of thyroxine-binding protein. I^{131} was rapidly concentrated by the gland, and administration of potassium thiocyanate did not cause the release of trapped iodide. Endogenously labeled material in the serum was 60–65 per cent insoluble in butanol. The soluble fraction proved to be thyroxine and triiodothyronine. Starch-block electrophoresis and ultracentrifugation identified the butanol-insoluble portion as an abnormal iodinated protein having the mobility of albumin. This protein was not precipitated by equine anti-human serum albumin. After enzymatic hydrolysis of plasma, mono- and diiodotyrosine, thyroxine, and triiodothyronine were identified on paper chromatograms.

This patient apparently suffered from a congenital thyroid abnormality which was characterized by the release of large quantities of a metabolically inactive iodinated protein into the serum. The protein may have been related to thyroglobulin. This abnormality may be similar to that previously observed in certain patients with carcinoma of the thyroid or thyroiditis.

METABOLIC defects in the thyroid gland associated with goiter and hypothyroidism include an inability to organify trapped iodide, a defect in deiodination of iodotyrosines, and possibly an impairment in conjugation of iodotyrosines (1). The present report describes another metabolic abnormality in a patient with congenital hypothyroidism, retarded development, and goiter. The serum contained an abnormal iodinated protein which was insoluble in butanol. Upon enzymatic hydrolysis, this protein yielded monoiodotyrosine, diiodotyrosine, and thyroxine.

CASE REPORT

E.J. (M.G.H. #429655), a 28-year-old single white female, entered the Massachusetts General Hospital for the first time in 1944 at the age of 15 because of gradual enlargement

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of the thyroid gland. Although she was apparently normal at birth, retarded growth was soon observed. She did not walk until she was 2 years old and did not talk until she was 3. At age 8 a goiter was first noted, but no treatment was given. There was no history of abnormal diet or goitrogenic medication. She was short, obese and dull, with the facies of a mild cretin and a firm, nodular thyroid gland five times normal size. Roentgenograms demonstrated a normal skull and a bone age of 13.5 years. Determinations of the basal metabolic rate ranged from −12 to −22 per cent. The serum cholesterol concentration was 329 mg. per 100 ml. Subtotal resection of the goiter was performed. "Multiple fetal adenomata" were reported after pathologic examination of the specimen. After discharge she received a maintenance dosage of desiccated thyroid. She became more alert and was relatively well adjusted at her performance level. Menses continued normally, and breast development was, if anything, excessive.

When the patient returned to the Thyroid Clinic in 1956 it was recognized that she might be a "metabolic cretin." The maintenance dosage of 2 grains of desiccated thyroid daily was discontinued for a period of ten weeks, and she was admitted to the metabolic ward. There were no symptoms, other than constipation, that might be attributed to hypothyroidism.

The patient was a short (136 cm.), obese (59.4 Kg.), retarded adult. Blood pressure was 105/85, respirations 12 per minute, temperature 98° F., and pulse rate 72 per minute. Her tongue was larger than normal, and the skin was dry though not cool. Hands and feet were puffy. Axillary and pubic hair was sparse. The breasts were massive. Deep reflexes were normal. The thyroid gland was estimated to be twice the normal size. Determinations of the basal metabolic rate were −14 and −36 per cent. Serum cholesterol concentrations were 326 mg. and 348 mg. per 100 ml., and protein-bound iodine concentration (PBI) was 2 μg. per 100 ml. The 24-hour uptake of I^{131} by the thyroid was 57 per cent. Review of the tissue sections prepared at the time of the patient's first admission revealed marked adenomatous hyperplasia with a histologic pattern similar to that found in other cases of congenital goiter seen in this clinic (2).

METHODS AND RESULTS

Thyroidal uptake, retention and release of iodine

After intravenous administration of 500 μc. of radioactive iodine (I^{131}), uptake by the thyroid was 57 per cent at twenty-four hours, 59 per cent at forty-eight hours, and 43 per cent at seventy-two hours. Serial determinations of the thyroidal I^{131} content for twenty-one days revealed an iodide release "half-time" of eighteen days. The total I^{131} concentration in the serum at forty-eight hours was 1 per cent of the dose per liter. Seventy-three per cent of the total radioactivity was precipitable with trichloracetic acid. In another study, 10 μc. of I^{131} was given orally. After two hours 27 per cent had accumulated in the thyroid, and administration of 1 gram of potassium thiocyanate failed to effect a release of I^{131} from the gland.

Fate of labeled thyroxine and diiodotyrosine

After intravenous administration of 66 μc. of I^{131}-labeled thyroxine (containing approximately 5 μg. of stable thyroxine), serial observations were made on blood and urine. The extrapolated initial volume of distribution

was 11.1 liters, or 18.6 per cent of the body weight. The plasma thyroxine "half-life" was 8.5 days. Chromatograms of serum demonstrated labeled thyroxine and small amounts of iodide, whereas urine contained only labeled iodide.

A dose of 50 μc. of I^{131}-labeled *l*-diiodotyrosine (86.4 per cent labeled diiodotyrosine, 13.6 per cent labeled iodide) was administered intravenously, and its urinary disposal observed in the manner previously described (3). In the first hour 18 per cent of the dose was excreted, of which 85 per cent was iodide; in the second hour 10 per cent was excreted, with 98 per cent as iodide; and in the fourth hour 10 per cent was excreted, with 99 per cent as iodide.

Characterization of the I^{131}-labeled compounds in serum

a. *Solubility.* Labeled iodine appearing in the serum after administration of I^{131} was only partially extractable by n-butanol. Serial specimens were acidified with sulfuric acid to pH 2 and extracted once with butanol saturated with 5 per cent sodium thiosulfate in water, and thrice with water-saturated butanol. Over the first few days the butanol-inextractable fraction increased to a maximum of 60–65 per cent of the total and remained constant for a total period of observation of twenty days. In normal control plasma 90 per cent or more of blood I^{131} was butanol-soluble under the conditions indicated. Likewise, I^{131}-labeled thyroxine added to normal serum or to the serum of the patient under discussion was over 90 per cent extractable.

Alkalinization or heat denaturation of the serum prior to butanol extraction, or extraction of acidified serum with methanol or ether, did not increase the recovery of I^{131}. Mixing serum with an equal volume of normal serum did not augment the butanol extractability. However, prior precipitation of the plasma proteins with either 20 per cent trichloracetic acid or with Somogyi's reagent increased the butanol extractability of the labeled compounds from 10–20 per cent to 55–75 per cent.

b. *Chromatographic identification.* Chromatograms were made of untreated sera and butanol extracts. The latter were prepared by acidifying the serum sample to pH 2 and extracting three times with 4 volumes of butanol. The extract was evaporated to dryness under an air stream, and the residue re-extracted with ethanol-ammonia (4:1). The ethanol-ammonia extracts were applied to strips of Whatman #3 filter paper (4 cm. wide) and the chromatograms developed in descending fashion either in butanol-acetic acid or butanol-isoamyl-ammonia systems. Known compounds were added to each strip as markers. After staining the markers, appropriate segments were cut out and radioactivity determined in a well-type scintillation counter with a sensitivity of 3×10^5 cpm/microcurie.

Enzymatic hydrolysis of serum was carried out in the following ways:

1) Crude trypsin[1] was added to serum in a concentration of 10 mg./ml. The mixture was adjusted to pH 8.0–8.5 with carbonate-bicarbonate buffer and incubated for twenty-four to forty-eight hours at 37° C.

2) Trypsin digestion of serum for twenty-four hours was followed by addition of chymotrypsin[2] (5 mg./ml.), and the mixture was reincubated for another twenty-four hours.

3) Chymotrypsin was added to serum in a concentration of 5 mg./ml., the mixture brought to pH 8.0–8.5 with carbonate-bicarbonate buffer, and incubated for twenty-four to forty-eight hours at 37° C.

4) Pepsin[2] was added to serum in a concentration of 10 mg./ml., the pH adjusted to 2 with 5*N* HCl, and digestion carried out for twenty-four to seventy-two hours.

During the course of these hydrolyses, the pH was measured and adjusted to the starting level, if necessary.

After enzymatic digestion, butanol extractability of the I^{131}-labeled compounds increased. In a typical sample with 60 per cent of the radioactivity protein bound, 37 per cent was butanol-insoluble. After trypsin digestion, however, 20 per cent was not extractable in butanol. After trypsin-chymotrypsin, 13 per cent was butanol-insoluble; after chymotrypsin digestion, 10 per cent; and after pepsin digestion, 7 per cent was inextractable. Concomitant with the increase of butanol-soluble material and the decrease in residual radioactivity in the protein layer, there was an increase in material which was water-soluble but not butanol-soluble. This water-soluble iodinated component was most prominent in the pepsin digests, where it accounted for up to 13 per cent of total sample radioactivity. This fraction, presumably formed during hydrolysis, was not fully characterized because of the minute amounts present, but was thought to be a short-chain polypeptide.

Chromatograms of butanol extracts of serum hydrolysates revealed monoiodotyrosine (MIT) and diiodotyrosine (DIT) in addition to iodide and thyroxine-triiodothyronine (T_4-T_3). (Usually, chromatographic separation of T_4 and T_3 was not carried out, so no statement as to their proportional content can be made.) The distribution of radioactivity in typical chromatographic studies is shown in Table 1. Despite variability due to error imposed by the low counting rates, repeated determinations followed the same pattern. Enzymatic hydrolysis of *normal* serum in the presence of I^{131} did not result in the appearance of labeled thyroxine or iodotyrosines, and enzymatic hydrolysis of normal serum in the presence of I^{131}-labeled-*l*-thyroxine did not result in the appearance of labeled iodotyrosines.

[1] Armour Laboratories, Chicago, Illinois.

[2] Worthington Biochemical Corporation, Freehold, New Jersey.

TABLE 1. DISTRIBUTION OF IODINATED COMPOUNDS IN SERUM OF PATIENT E.J.

Time after I^{131}	Material analyzed	Total bkgrnd. counts	Net total counts	% Total serum radioactivity as				
				Origin	Iodide	MIT*	DIT*	T_{3-4}*
3 hours	Untreated serum	20	6482	0	97	.2	0.5	1.0
2 days	Butanol extract	20	662	0	51	0.1	0.1	21
11 days	Untreated serum	113	123	50	0	8	3	30
4 days†	Butanol extract of trichloracetic acid precipitate	38	525	0	4	4	2	86
4 days†	Butanol extract of hydrolysates:							
	Trypsin	85	1327	0	36	9	6	43
	Trypsin + chymotrypsin	77	868	0	29	17	12	36
	Chymotrypsin	78	1984	0	31	30	14	21
	Pepsin	62	1160	0	23	23	23	12
4 days†	Butanol extracts of hydrolysates of nonbutanol-extractable residue:							
	Trypsin	80	618	0	14	17	26	33
	Trypsin + chymotrypsin	80	541	0	15	32	21	37
	Chymotrypsin	83	1704	0	6	7	8	68
	Pepsin	93	1440	0	7	27	23	38

* MIT = Monoiodotyrosine.
DIT = diiodotyrosine.
T_{3-4} = thyroxine-triiodothyronine.

† This serum sample contained 0.19% dose/L. as total I^{131}, 0.12% dose/L. as PBI^{131}, and was 37% butanol-insoluble. It contained previously injected I^{131}-labeled thyroxine providing 30% of total radioactivity. In all calculations on this sample, radioactivity attributable to previously injected I^{131}-labeled thyroxine (after allowance for physical and biologic decay) was subtracted before calculation of % dose I^{131}/L., % dose PBI^{131}/L., and % radioactivity as endogeneously formed thyroxine.

c. *Transport of iodinated components.* Two 2-ml. samples of serum, drawn four and eleven days respectively after the initial tracer dose of I^{131}, were fractionated by electrophoresis in a starch-supported barbital buffer at pH 8.6 (4). In both samples radioactivity was identified primarily as a single zone associated with the peak and the advance limb of the albumin fraction (Fig. 1A). Twelve hours after intravenous injection of 66.5 μc. of I^{131}-labeled *l*-thyroxine, radioactivity was localized between the albumin and α-globulin zones, presumably in association with the normal thyroxine-binding globulin (Fig. 1B). A second I^{131} tracer was administered thirteen days after the injection of labeled thyroxine. Serum obtained four days later had two peaks of radioactivity (Fig. 1C). These correspond to the single peaks demonstrated after I^{131} and labeled thyroxine alone (Figs. 1A and 1B, respectively), testifying to the separability of the normal thyroxine carrier from the patient's endogenously labeled material under these conditions.

The capacity of the patient's serum to bind exogenous thyroxine was normal or increased. Serum specimens were studied by paper electrophoresis after fifteen minutes of incubation *in vitro* with I^{131}-labeled *l*-thyroxine. Thyroxine was added in graded quantities to yield serum con-

centrations of 12, 60, and 120 μg. of added thyroxine per 100 ml. Simultaneous determinations were made on duplicate samples of normal serum at each thyroxine level. As shown in Figure 2, added thyroxine was transported predominantly in the zone between α_1 and α_2 globulin, even when the serum concentration of thyroxine was 120 μg. per 100 ml. By contrast, the preponderance of radioactivity was associated with albumin at this thyroxine concentration in normal serum. The apparent increase in the quantity of thyroxine-binding globulin or its affinity for thyroxine was not accompanied by any significant abnormality in the distribution of plasma proteins as determined by free boundary electrophoresis at pH 8.6 in barbital buffer.

The patient's labeled serum, collected seven days following administration of 500 μc. of I^{131} and twenty days following administration of 66 μc. of I^{131}-labelled thyroxine, was spun in a Spinco Model E ultracentrifuge at 56,100 rpm for ten hours. After the proteins migrated past the diaphragm, the portions above and below were removed separately. The protein layer

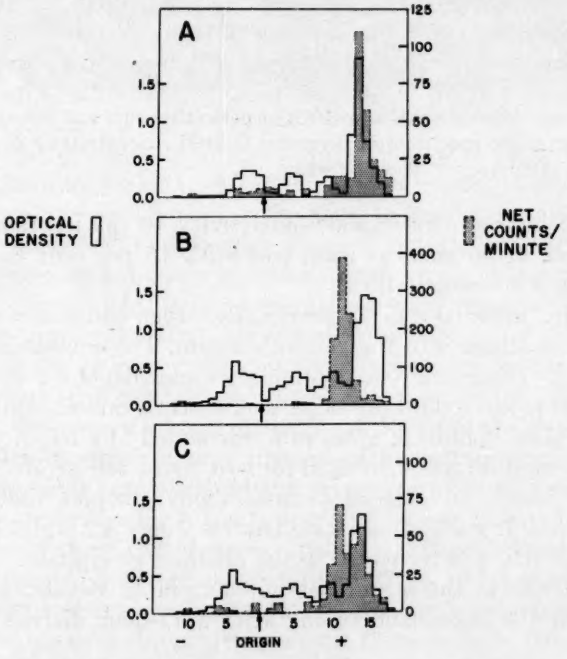

FIG. 1. Starch block electrophoresis of patient's serum at pH 8.6: **A.** Four days after I^{131}. **B.** Half a day after I^{131}-labeled *l*-thyroxine administered intravenously. **C.** Four days after a second tracer dose of I^{131} (seventeen days after the administration of labeled thyroxine).

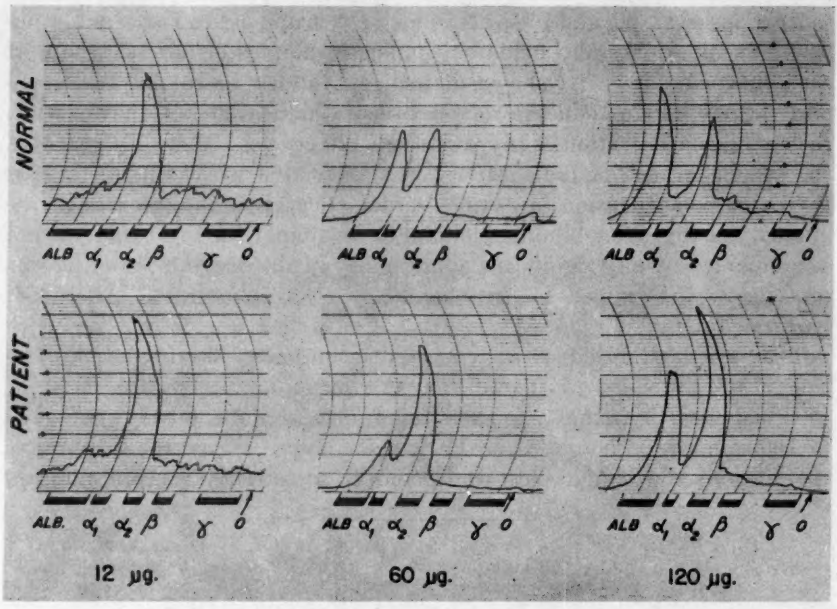

FIG. 2. Paper electrophoresis at pH 8.6 of patient's serum and normal serum after addition of increasing quantities of thyroxine to yield concentrations of 12 μg., 60 μg. and 120 μg. of thyroxine per 100 ml. of serum.

contained 88 per cent of the total radioactivity. Of this fraction 41 per cent was insoluble in butanol, as compared with 13 per cent insolubility in butanol of the nonprotein layer.

The labeled material was not precipitated upon addition of equine anti-human serum albumin to the patient's serum. The equivalence zone was established by observing precipitation after addition of a constant volume of antiserum to serial dilutions of patient's serum. Subsequently the calculated equivalent volume of antiserum was added to 2 ml. of the patient's serum. The mixture was incubated for two hours, refrigerated at 4° C. for forty-eight hours, and the albumin-antibody complex then settled by centrifugation. In each of duplicate analyses, only 4.2 and 3.7 per cent of the radioactivity was recovered in the albumin precipitate.

Radioactivity in the serum was not dialyzable. Seventy-five per cent remained in the cellophane tubing after a 72-hour dialysis against tap water.

DISCUSSION

A case of congenital hypothyroidism has been described and an abnormal iodinated compound demonstrated in the serum. This material was pre-

cipitable with proteins, was nondialyzable, was sedimented with protein in the ultracentrifuge, and was insoluble in butanol. It moved with the serum albumin fraction upon electrophoresis but was apparently not part of, or necessarily associated with, serum albumin since it did not precipitate in the presence of albumin antiserum. Enzymatic hydrolysis liberated monoiodotyrosine, diiodotyrosine and thyroxine.

These findings are consistent with the postulate that the defect in this patient was the formation of an abnormal thyroprotein in the gland or abnormal fragmentation and release of normal thyroglobulin. The fact that trypsin did not completely hydrolyze this protein, in contrast to normal thyroglobulin, is evidence in support of the formation of an abnormal thyroprotein. Thyroglobulin is completely digested by trypsin under identical conditions (unpublished data of the authors).

This endogenously formed, iodinated protein did not move in the inter-alpha zone which is characteristic of thyroglobulin transport at this pH (5). Further, its presence after a 500-μc. radioactive tracer and its constant proportion in the serum over the observed period also argues against its being thyroglobulin released through a radiation effect. The radiation administered to the gland during the first four days, calculated on the basis of 58 per cent uptake, 18-day thyroidal "half-time," and a thyroid weight of 30 Gm., was 292 rad.

There was no evidence of a defect in trapping or binding of iodide, or inability to form thyroxine or deiodinate diiodotyrosine. Normal metabolism of labeled thyroxine and clinical response to desiccated thyroid exclude peripheral "resistance" to thyroxine as a factor in the patient's hypothyroid state. Goiter formation during the patient's youth, before replacement therapy was begun, demonstrated normal pituitary response to hypothyroidism. The low serum PBI^{127} during the study period was thought to represent a delay in hypertrophy of the thyroid and restoration of the endogenous PBI level. On the other hand, it is possible that the defect itself prohibited release of normal quantities of I^{127} from the gland.

Except for the presence of large amounts of diiodotyrosine, the abnormal substance described herein shared the properties of compound "X," observed in the serum of some patients with thyroid carcinoma (5, 6). It may be the factor in other cases of goiter in which iodinated compounds in the blood are incompletely extractable in butanol (7, 8), and is possibly related to the non-butanol-extractable fraction of the protein-bound iodine reported in patients with chronic lymphocytic thyroiditis (9, 10).

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METABOLISM OF FREE AND CONJUGATED 17-HYDROXYCORTICOSTEROIDS IN SUBJECTS WITH THYROID DISEASE*

HAROLD BROWN, M.D., EDWIN ENGLERT, JR., M.D.† AND STANLEY WALLACH, M.D.‡

The Veterans Administration Hospital and Department of Medicine, University of Utah College of Medicine, Salt Lake City, Utah

ABSTRACT

Levels of free and conjugated 17-hydroxycorticosteroids (17-OHCS) in plasma were measured after intravenous infusions of cortisol, tetrahydrocortisone (Tetra E) and adrenocorticotropic hormone (ACTH) in subjects wi h hyperthyroidism and hypothyroidism. After administration of cortisol to hyperthyroid patients, the levels of free 17-OHCS decayed more rapidly than normal, while those of conjugated 17-OHCS rose faster and to higher values than in normal subjects. The opposite occurred in hypothyroid patients. Conjugation of administered Tetra E was increased in hyperthyroid patients but was normal in those with hypothyroidism. Plasma concentrations of free 17-OHCS were influenced more by the rate of removal from plasma than by the adrenal response to ACTH in both types of subjects. Accordingly the level of free 17-OHCS after ACTH was a misleading index of adrenocortical function, as the rise was greatest in hypothyroid patients and least in those with hyperthyroidism. The level of conjugated 17-OHCS after infusion of ACTH was highest in hyperthyroid patients and lowest in hypothyroid patients; it reflected accurately the state of adrenocortical activity. The amount of unstimulated excretion of 17-OHCS in the urine was in keeping with this observation, the total output varying directly with basal metabolic rate. In patients restudied after appropriate therapy, the metabolism of 17-OHCS returned towards normal.

In hyperthyroidism the removal of cortisol from plasma and the formation of conjugated 17-OHCS occur more rapidly than normal, the reverse being true in hypothyroidism. Since conjugation of Tetra E appears to be normal in hypothyroidism, the apparent failure of conjugation after administration of cortisol probably results from decreased reduction of cortisol to substrates needed for conjugation. For proper assessment of adrenocortical function it is necessary to determine the rate of removal of 17-OHCS from plasma, as well as the response to ACTH, and the urinary excretion of these steroids. Results of such an analysis suggest that adrenocortical activity varies directly with basal metabolic rate.

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† Fellow, U.S.P.H.S. Present Address: Massachusetts Memorial Hospital, Boston 18, Mass.

‡ Present Address: Massachusetts General Hospital, Boston 14, Mass.

PREVIOUS studies have indicated that the metabolism of cortisol is influenced by the functional state of the liver (1), the kidneys (2) and the age of the subject (3, 4). Recently, it has been shown that the metabolic state produced by either hyper- or hypothyroidism may also influence the metabolism of cortisol (5, 6). Levin and Daughaday (5) have reviewed the relation between thyroid and adrenocortical function and reported their own observations. This communication will report observations on the metabolism of free and conjugated 17-hydroxycorticosteroids (17-OHCS) in a group of subjects with either hyperthyroidism or hypothyroidism.

MATERIALS AND METHODS

An intravenous infusion of cortisol (1 mg. per Kg. of body weight) was given as previously described (1) to 8 patients with hyperthyroidism, to 6 patients with hypothyroidism, and to a group of normal subjects. These patients were restudied when their metabolic state had been corrected. Samples of blood were drawn prior to the infusion and at half, one, two, four and six hours after the beginning of the infusion, which lasted about thirty minutes. The plasma was separated and stored frozen. The level of free 17-OHCS in the plasma was measured by the method of Nelson, Samuels and Eik-Nes (7, 8) and the level of conjugated 17-OHCS by a modification (9) of the method of Bongiovanni (10).

Three subjects with myxedema and 2 with thyrotoxicosis were given an infusion of tetrahydrocortisone containing 1 mg. per Kg. of body weight, and the levels of free and conjugated 17-OHCS in the plasma were measured serially (1). The infusions lasted two to three minutes.

The rise in the levels of plasma 17-OHCS in response to a standard infusion of ACTH (11) was measured in 3 patients with hyperthyroidism and in 4 patients with hypothyroidism. In most of these subjects, the studies were repeated when the basal metabolic rate (BMR) was restored to the normal range.

The 24-hour 17-OHCS excretion in the urine was measured in 10 subjects with hyperthyroidism and in 7 subjects with hypothyroidism by a modification of the methods of Glenn and Nelson (12) and Bongiovanni (10). Urine was stored in the refrigerator during the 24-hour collection periods and an aliquot was frozen for later analysis for total 17-OHCS. Duplicate samples estimated to contain 1–5 μg. of 17-OHCS were each mixed with 2 volumes of 0.2 *M* acetate buffer (pH 4.5) and 0.1 volume of a solution of β-glucuronidase (containing 5,000 U. per ml.) in a 50-ml. round-bottom centrifuge tube. The mixture was incubated at 37° C. for twenty-four hours. Thereafter, the procedure for the estimation of conjugated 17-OHCS in plasma was followed (9).

The diagnosis of myxedema or of thyrotoxicosis was made by conventional methods, using clinical appraisal, BMR, thyroidal uptake of I^{131}, and protein-bound iodine levels. Questionable cases were not studied.

RESULTS

Plasma free 17-OHCS after infusion of cortisol

The mean levels of free and conjugated 17-OHCS in the plasma following the infusion of cortisol are listed in Table 1, and the mean values of free 17-OHCS are represented in Figure 1. It is apparent that the disappearance

TABLE 1. EFFECT OF INFUSION OF CORTISOL ON PLASMA LEVELS OF FREE AND CONJUGATED 17-OHCS (μG./100 ML.)

	No. of subjects	Hours												Removal slope of cortisol (log conc. per hr.)	Half-life (mins.)
		0		0.5		1		2		4		6			
		Free	Conj.	Free	Conj.	Free	Conj.	Free	Conj.	Free	Conj.	Free	Conj.		
Hyperthyroid	8														
Mean		12.9	17.0	114	53	78	103	43	87	12	60	5	37	−0.2736	69
± S.E.M.		1.8	2.9	15.8	9.4	6.4	14.3	4.1	14.5	2.7	10.6	1.1	10.6	0.019	5
Hypothyroid	6														
Mean		16.8	12.8	139	29	90	34	68	37	46	33	36	25		
± S.E.M.		1.1	2.6	11	4.2	4.9	3.1	3.8	5.1	4.7	3.9	4.7	2.6		
Treated Hyperthyroid	6														
Mean		11.6	7.2	119	24	84	45	52	53	23	41	13	22	−0.1684	107
± S.E.M.		2.8	1.7	21.8	3.1	18.8	7	10.1	13	5	10	4	4	0.0314	19
Normal Series (9)	14														
Mean		12	12	119	32	83	45	57	53	28	41	13	31	−0.1618	112
± S.E.M.		0.7	1.6	9.3	3.3	2.9	3.8	2.3	3.5	1.4	3	1	2	0.0065	5

of free 17-OHCS from the plasma after infusion of cortisol was a logarithmic function for the patients with hyperthyroidism and for the treated hyperthyroid subjects. However, the exponential curve for the hyperthyroid group was much steeper than the curve for the treated group and passed through the half-hour level, whereas the curve of the treated group did not decline in a logarithmic fashion until one hour after the beginning of the cortisol infusion. The curve depicting the 17-OHCS levels of the

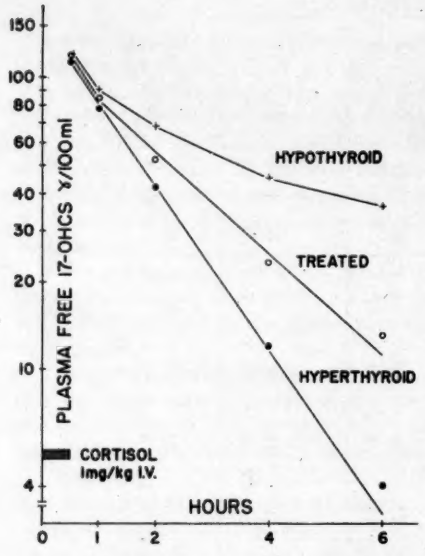

FIG. 1. The mean levels of free 17-OHCS in the plasma after infusion of cortisol. (The data are listed in Table 1.)

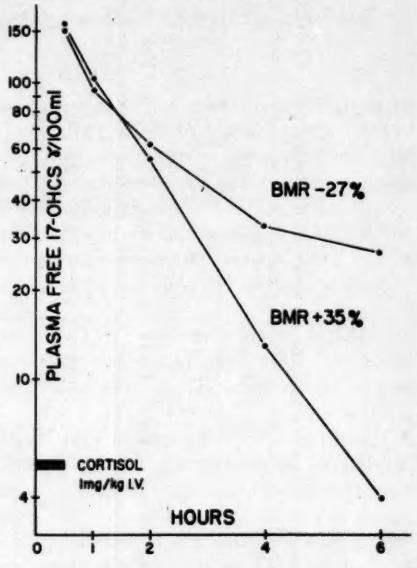

FIG. 2. Effect of antithyroid treatment on the removal of free 17-OHCS from plasma, in a young man (G.S.) who was inadvertently overtreated for hyperthyroidism.

subjects with hypothyroidism was not a straight line for at least the first four hours.

The mean slope of the line and the standard error of the mean was $-0.274 \pm .019$ for the thyrotoxic group as compared with -0.162 ± 0.007 for a group of normal subjects (9). The difference between the slopes for the thyrotoxic and for the euthyroid subjects was significant ($P < .01$). After the thyrotoxic patients were treated, the slope of the cortisol disappearance curve fell within the normal range, the value being -0.168 ± 0.031.

The calculated half-time of disappearance of free 17-OHCS from the

plasma of the subjects with hyperthyroidism was 69 ±5 minutes and after treatment it was 107 ±19 minutes.

The changing rates of disappearance of cortisol in an individual patient are illustrated in Figure 2, which depicts serial studies made on a young man (G.S.) when he was hyperthyroid and also when he was hypothyroid as a result of inadvertent overtreatment.

Plasma conjugated 17-OHCS after infusion of cortisol

The levels of conjugated 17-OHCS in the plasma after the infusion of cortisol were much more variable than those of free 17-OHCS, but showed a definite pattern. In the treated and normal subjects, the concentration of conjugated 17-OHCS in the plasma rose to a peak at two hours and then slowly declined (Fig. 3). In the patients with hyperthyroidism, the concentration of conjugated 17-OHCS in the plasma rose more quickly and to much higher levels than in the normal group ($P < .005$). In contrast, the levels in the patients with hypothyroidism were significantly lower at their peak than in the normal subjects ($P < .02$). In both groups, after the patients had been treated, the levels of conjugated 17-OHCS tended to reach normal values. The concentration of conjugated 17-OHCS in the plasma of both normal (9) and thyrotoxic subjects fell in an exponential fashion. The rate of fall was more rapid than normal in the patients with thyrotoxicosis and less rapid in those with myxedema. The levels of conjugated 17-OHCS in the plasma of Patient G.S., who was studied when his BMR was +35 per cent and again when it was −27 per cent, are shown in Figure 4.

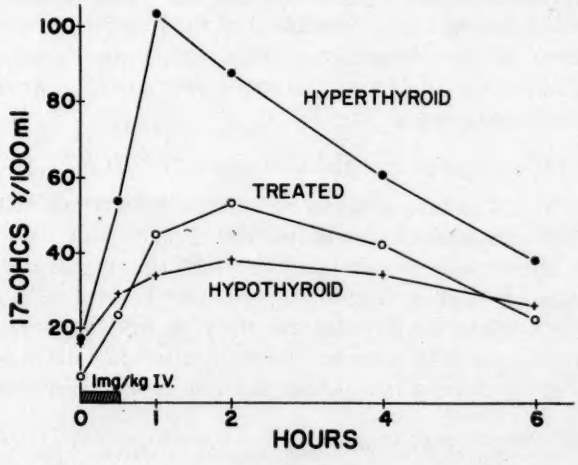

FIG. 3. The mean levels of conjugated 17-OHCS in the plasma after the infusion of cortisol. (The data are given in Table 1.)

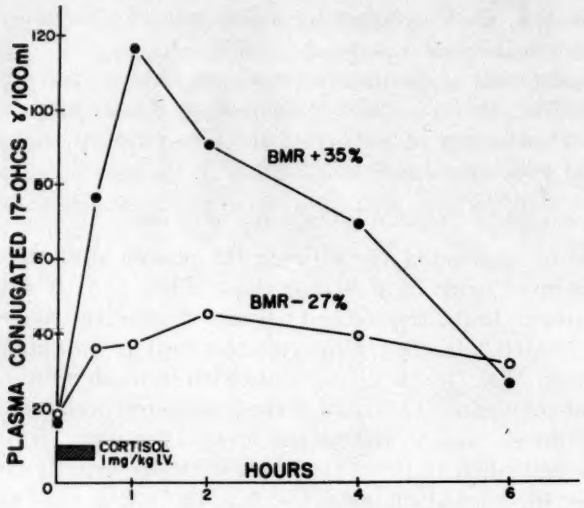

FIG. 4. The levels of conjugated 17-OHCS in the plasma of Patient G.S. after infusion of cortisol. (See Fig. 2.)

Urinary 17-OHCS excretion

The mean daily excretion of 17-OHCS in the urine of a group of 10 normal males, as measured by the method employed in this study, was 8.9 mg. (range 6.2–13.1). In the patients with hyperthyroidism it was 20.7 mg. and in those with hypothyroidism, 3.2 mg. In all instances, the 17-OHCS excretion declined after treatment of the hyperthyroidism and rose after treatment of the myxedema. There was a significant correlation ($r = .82 \pm .12$) between the basal metabolic rate and the amount of 17-OHCS excreted in the urine (Fig. 5).

Effect of ACTH on plasma free and conjugated 17-OHCS

The effect of a standard infusion of adrenocorticotropin (ACTH) (11) on the free and conjugated plasma 17-OHCS levels was studied in 7 instances. In 5, there was an opportunity to repeat the studies after the BMR had been altered by appropriate therapy. It can be seen (Fig. 6) that the free 17-OHCS levels in the hypothyroid patients rose higher than those in the hyperthyroid patients, whereas the conjugated 17-OHCS levels in the hypothyroid group did not rise as high as those in the hyperthyroid group.

Effect of tetrahydrocortisone on plasma free and conjugated 17-OHCS

The levels of plasma free 17-OHCS in the patients with hyperthyroidism fifteen minutes after the beginning of infusion of tetrahydrocortisone were

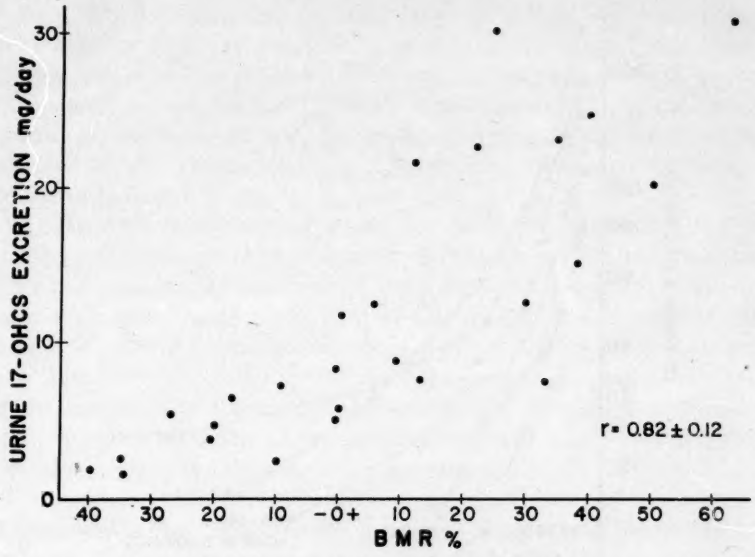

FIG. 5. The relation between the BMR and the 24-hour urinary excretion of 17-OHCS in the patients with thyroid disease before and after treatment.

not as high as in the normal subjects (9) or in those with hypothyroidism. The free 17-OHCS levels in the plasma of all subjects declined exponentially for about two hours. After this there appeared to be a delay in removal of 17-OHCS from the plasma of the patients with hypothyroidism (Fig. 7).

As compared with the normal subjects, the patients with hypothyroidism

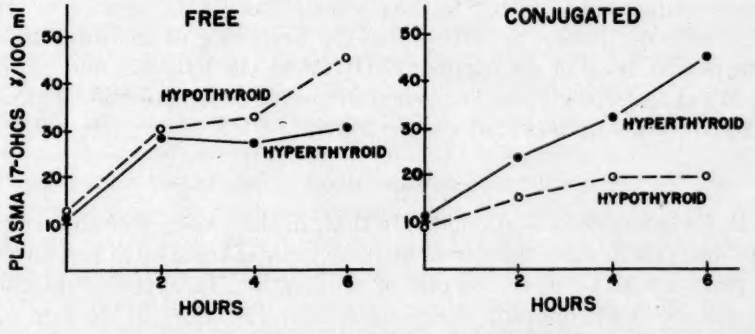

FIG. 6. The mean levels of free and conjugated 17-OHCS in the plasma of 3 patients with hyperthyroidism (●——●) and 4 patients with myxedema (○ – – ○) during a standard infusion of ACTH.

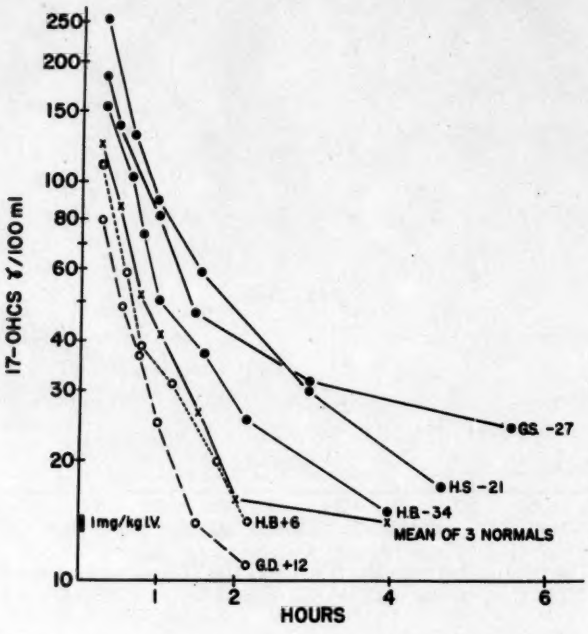

FIG. 7. The mean levels of free 17-OHCS in plasma after an infusion of tetrahydrocortisone (1 mg./Kg. body weight) in 3 patients with hypothyroidism (●——●) and 2 patients with hyperthyroidism (○ – – ○) compared with the mean levels in 3 normal subjects (×——×). In all cases the infusion lasted two to three minutes.

manifested no impairment in their ability to form conjugated 17-OHCS, whereas the hyperthyroid patients again displayed an increased capacity to form conjugated 17-OHCS in the plasma following the infusion of tetrahydrocortisone. Fifteen minutes after the beginning of the infusion, the mean plasma level of conjugated 17-OHCS in the hyperthyroid patients was 245 μg. per 100 ml., in the normal subjects 128 μg. per 100 ml., and in the hypothyroid patients 161 μg. per 100 ml.

DISCUSSION

The foregoing data would indicate that, in the presence of an increased metabolic rate in man, there is an increased rate of removal of cortisol from the plasma with an increased rate of conjugation. In hypothyroidism the changes are in the opposite direction. Similar variations in the rate of removal of free 17-OHCS have also been noted by others (5, 6).

It is of interest that in the hyperthyroid patients the level of free 17-OHCS at thirty minutes after the beginning of the cortisol infusion fol-

lowed a straight line when the logarithm of the level was plotted against time, whereas this was not true in normal subjects until one hour had elapsed (Fig. 1). The rapid equilibration in the patients with hyperthyroidism may be attributable to more rapid mixing. In the patients with myxedema, the decay of free 17-OHCS in the plasma was not an exponential function. This suggests that, in myxedema, there may be more than one rate-limiting reaction.

In view of the important role of the liver in the metabolism of 17-OHCS, it would seem reasonable to explain these changes in 17-OHCS metabolism on the basis of variations in hepatic blood flow. In the dog it has been possible to effect more rapid removal of 17-OHCS from the plasma and to increase the rate of formation of conjugated 17-OHCS by increasing the blood flow to the liver, and the opposite has been accomplished by decreasing the hepatic blood flow (13). Measurements of splanchnic blood flow in patients with myxedema have not been reported (14), but it may well be reduced in view of the decreased cardiac output (15), renal blood flow (16) and cerebral blood flow (17) in this condition. Myers and coworkers (18) have shown that in thyrotoxicosis in man the splanchnic blood flow is not increased, although the oxygen extraction is higher than in normal subjects. Diminished hepatic blood flow could play a role in patients with myxedema, but it is more likely that variations in the enzymatic activity of the cells of the liver and possibly of other tissues account for the variations in the 17-OHCS metabolism in thyroid disease. This concept is supported by several experimental observations. Brown noted that cortisone was metabolized more rapidly in liver slices from rats pretreated with triiodothyronine than in liver slices from untreated animals (19). Again, Kumagai, Freinkel and Ingbar found that in hyperthyroid and hypothyroid adrenalectomized mice the sensitivity of the target organs to cortisone varied inversely with the BMR (20).

It is of considerable interest that the variations in cortisol metabolism from species to species and at different ages in man correlate well with the metabolic rate. For example, the rate of removal of cortisol and the metabolic rate are faster in children than in adults (4), faster in the adult than in the aged (3) and faster in the dog than in man (13, 21).

The plasma levels of free 17-OHCS during stimulation with ACTH were lower when the patients were hyperthyroid than when they were in the euthyroid state. The opposite occurred in hypothyroid patients. This response to ACTH does not imply an inverse relationship between thyroidal activity and adrenocortical capacity, but is probably an indication that factors controlling removal overshadow those leading to accumulation of plasma free 17-OHCS. This would account for the increased levels of conjugated 17-OHCS in the plasma during ACTH stimulation in subjects

with an elevated BMR. By means of a mathematical analysis (22) which took account of the rate of removal as well as the increased levels of 17-OHCS in the plasma produced by ACTH stimulation, Samuels (23) has calculated from the data of Patient G.S. (Fig. 4) that his "adrenal capacity"—that is, how much 17-OHCS was produced with the standard ACTH test—was 139 μg./Kg./hour of 17-OHCS when his BMR was −27 per cent and 183 μg./Kg./hour when his BMR was +35 per cent. Similarly, the calculated production of 17-OHCS to maintain the 8 A.M. level during these periods would be 10.1 μg./Kg./hour and 15.2 μg./Kg./hour, respectively. These analyses suggest that adrenocortical capacity is directly related to thyroidal activity.

This again points out the difficulty of assessing adrenocortical function by the results of a single test, even the response of the plasma 17-OHCS to ACTH, without reference to other factors of 17-OHCS metabolism, such as the rate of removal from the plasma.

The excretion of 17-OHCS in the urine was in keeping with the foregoing observations. The amount of 17-OHCS excretion varied directly with the BMR, both in individual subjects who were studied before and after therapy and when one subject was compared with another. This implies that more 17-OHCS were being produced, possibly in an effort to maintain plasma levels in the face of accelerated removal of the 17-OHCS from the plasma. Some of the hyperthyroid subjects excreted amounts of 17-OHCS in the urine comparable to those seen in Cushing's syndrome. The diminished 17-OHCS excretion in the urine of patients with myxedema resulted in part from the markedly reduced glomerular filtration (16) in this condition and the decreased formation of conjugated 17-OHCS. It has been established that the principal excretory product of the 17-OHCS is the conjugated material (24) which is excreted into the urine by glomerular filtration (25).

It is interesting to speculate concerning the effects of thyroid storm on 17-OHCS metabolism. There are many similarities between the symptomatology of this syndrome and that of acute adrenocortical insufficiency. It is possible that the exaggerated rate of removal of the 17-OHCS from the plasma and the increased requirement of 17-OHCS under the conditions of thyroid storm may result in relative adrenocortical insufficiency despite the increased production by the adrenal cortex. Rawson and Rall (14) reported dramatic results in 5 patients with thyroid storm who were given aqueous adrenocortical extract in doses comparable to those given in the treatment of Addisonian crisis.

The results of the studies with tetrahydrocortisone indicate that the deficient conjugation of 17-OHCS by the hypothyroid patients when given an infusion of cortisol is more apparent than real. When tetrahydrocorti-

sone was available to these patients, conjugation proceeded at a normal rate. However, after an infusion of cortisol, there was a delay in the conversion of cortisol to the reduced 17-hydroxycorticosteroids which are the substrate for glucuronide conjugation. Inasmuch as hepatic blood flow probably is reduced in hypothyroidism and yet the rate of conjugation of tetrahydrocortisone is not impaired, it is suggested that this degree of reduction in hepatic blood flow is not a limiting factor in the conjugation reaction. Similar results have been obtained in our laboratory in patients with cirrhosis of the liver (26). In these subjects there was decreased conjugation after administration of cortisol but a normal rate of conjugation after administration of tetrahydrocortisone. The increased rate of conjugation in the patients with hyperthyroidism is probably the result of the action of enzymatic factors. The increased levels of conjugated 17-OHCS in the patients with myxedema following infusion of tetrahydrocortisone could be the result of the decreased removal of conjugated 17-OHCS from the plasma consequent to the markedly reduced glomerular filtration in this condition (16).

It should be emphasized that, although we have demonstrated certain quantitative differences in the metabolism of the 17-OHCS in patients with thyroid disorders, we have not ruled out the possibility of qualitative differences. In view of the striking effect of thyroid function on enzymatic processes, the relative proportions of cortisol which are metabolized to reduced 17-OHCS, 20-hydroxylated compounds, 17-ketosteroids and 21-desoxy products may differ from those in normal subjects. Perhaps even other metabolites may be found. Isotopic and chromatographic techniques should prove fruitful in the study of the qualitative aspects of cortisol metabolism in thyroid disease.

Acknowledgments

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SERUM INORGANIC PHOSPHORUS LEVELS IN EUNUCHISM AND IN FEEBLE-MINDED STATES*

JAMES B. HAMILTON, PH.D. AND LEITHA D. BUNCH, M.A.

Department of Anatomy, State University of New York College of Medicine at New York City, Brooklyn, N. Y.; and H. L. Snyder Memorial Research Foundation, Winfield, Kansas

ABSTRACT

Serum inorganic phosphorus (SIP) concentration was typically and significantly higher in a series of 58 eunuchs than in 50 intact males of comparable age, mental status and environment. Throughout the entire span of age under study, 18 to 79 years, values were higher in eunuchs than in the intact control series, indicating that the waning testicular secretions of middle-aged and old men are adequate to depress the level of SIP.

Values for SIP concentration decreased with age in eunuchs. Therefore, in the absence of the restraint exerted by testicular secretions, SIP did not escape in old subjects to levels that were as high as in young adults.

As tested in eunuchs 20 to 39 years of age, two decades in which mean levels of SIP were similar, values for SIP were not significantly related to duration of the castrate state (three to twenty-five years) or age at orchiectomy (12 to 29 years). SIP values were not correlated significantly with those for secondary sex characters or for other structures and functions known to be affected by orchiectomy. Elevation of SIP levels in eunuchs was not due to a decrease in adrenocortical functions insofar as these can be gauged by titers of urinary 17-hydroxycorticosteroids and 17-ketosteroids. Indirect evidence suggests that the supranormal levels of SIP in eunuchs were not due solely or usually to increased stimulation by growth hormone.

SIP concentration tended to be subnormal in intact males who were feeble-minded, but it could rise after castration to levels in excess of those in intact men who were not feeble-minded.

IN ONE of our previous studies it was shown that the mean values for serum inorganic phosphorus (SIP) increased significantly within a few days after orchiectomy in 9 adolescent and young adult males and remained elevated during four years of subsequent study (1). In intact men

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and women (2), however, SIP did not become elevated significantly in middle and advanced age—a period when testicular and ovarian secretions decline materially, even to the extent that titers of urinary ketosteroids become as low as in young eunuchs. That values for SIP are lower in old than in young subjects, upon reduction of gonadal secretions, suggests one or both of the following: (a) SIP is unable to escape to markedly high levels in old subjects, or (b) the waning gonadal secretions in middle and old age continue to be adequate to depress SIP. To bear upon these and other questions, specific attention was paid in the present study to middle-aged and old eunuchs, and the degree to which SIP is influenced by age at orchiectomy or the duration of the castrate state. Values for SIP were also compared with those for other items influenced by testicular secretions; with measurements of various constituents of blood; and with titers of urinary metabolites of adrenocortical secretions, since subnormal secretion by these glands is associated with high SIP (3).

CLINICAL MATERIAL AND METHODS

Subjects

Eunuchs in this and the earlier study (1) were feeble-minded. Therefore a control series was employed, consisting of 50 feeble-minded intact males of comparable age (18 to 81 years) who lived in the same institution side by side with the eunuchs, followed a similar routine of life, and had access to an identical diet. Intact and castrate groups were roughly alike in genetic background, since the subjects admitted to this institution for the feeble-minded were drawn from the same population, chiefly of English and German extraction.

It was also considered desirable to ascertain if SIP was influenced by feeble-mindedness, a condition associated not only with abnormalities in the nervous system but also with low values in quantitative measurements of such target organs of gonadal secretions as axillary hair, beard and sebaceous glands (4). Accordingly, SIP values in the 50 feeble-minded intact males were compared with those in 110 normal intact males of comparable age.[1] Fifty-three of the intact males of normal mentality lived in the same community as the feeble-minded and were from a comparable ethnic population. The remaining 57 normal subjects were also Caucasian but lived in another part of the U.S.A. (2); they were an institutionalized group whose activities were restricted as in the case of feeble-minded subjects.

Many different types of feeble-mindedness were represented in the eunuchs and their intact control group. Subjects were chosen at random from those admitted to the institution, and were not excluded from study unless they could not, or would not, provide complete collections of urine when housed in rooms where loss of urine would be detected.

All subjects appeared to be in good health throughout the study as far as could be judged by results of physical examination and analyses of blood and urine. Orchiectomy had been carried out for legal reasons, not as therapy for diseases such as prostatic cancer. Illnesses have interfered with most attempts to assess the effects of castration, since

[1] Data for the adults of normal mentality (2) and for 9 of the eunuchs (1) have been reported previously.

disease is accompanied by low titers of 17-ketosteroid (5). The present study is complicated by the fact that the subjects were feeble-minded; however, the effects of impaired mentality tend to cancel out, as both the eunuchs and their intact control group were equivalent in this regard.

Methods

SIP concentration was measured by the technique described by Fiske and SubbaRow (6), adapted for use with a photoelectric colorimeter. With 2 exceptions the values for each subject represent an average of 2 to 14 (median of 3) determinations, utilizing separate collections of blood under basal conditions.

Serum calcium concentration was not studied, since it does not change significantly as a result of castration in man (1).

Urine was collected and assayed separately for two to seven periods of twenty-four or forty-eight hours. Determinations were made of 17-ketosteroids (7), 17-hydroxycorticosteroids (8), and creatinine (9).

Under basal conditions blood was withdrawn from an antecubital vein on two or more days. On each sample determinations were made in duplicate of the sedimentation rate (10) and fragility (11) of erythrocytes; concentration of hemoglobin (12); hematocrit (13); and the levels of serum alkaline phosphatase (method of King and Armstrong) (14), lipid phosphorus (15), alpha- and beta-lipoproteins (16), and total cholesterol. Total cholesterol was extracted from serum with a mixture of alcohol and ether (17), then dissolved in glacial acetic acid (14, p. 43) and determined by the Liebermann-Burchard color reaction as specified by Saifer (18).

Hair grown for known intervals following previous shaving was collected from the right axilla for two or more periods of six to nine weeks, and from the beard area for three or more periods of forty-eight hours. Reliable methods for obtaining, processing and weighing samples of hair have been developed (4). Sebum was measured by a method similar in principle to that described by Jones *et al.* (19).

Measurements of the intelligence quotient in the feeble-minded were provided by psychologists employed by the State of Kansas, who used Form I of the Wechsler-Bellevue test and Form L of the Revised Stanford-Binet test.

RESULTS

Statistical analyses of values obtained on serial samples from the same subjects showed that these values could be duplicated upon repetition with the methods employed.

Higher values for SIP in eunuchs than in intact males, as tested throughout the lifespan in feeble-minded subjects

The SIP level was typically and significantly higher in eunuchs than in intact but otherwise comparable males living under similar circumstances (Table 1 and Fig. 1). There was little overlap at any age in individual values between castrated and intact males. There was, however, a considerable range in values for the same person from time to time. This was masked, since the level of SIP shown for a subject is the average of several tests.

The mean difference for SIP between castrated and intact males (Table

TABLE 1. COMPARISON OF SERUM INORGANIC PHOSPHORUS LEVELS IN EUNUCHS WITH THOSE IN INTACT BUT OTHERWISE COMPARABLE MALES

Age (yrs.)		Subjects		SIP (mg./100 ml.)					
Range	Mean	Status	No.	Mean	P	Median	Range	S.D.*	C.V.†
18–38	31.5	Castrate	35	4.17	<.001	4.09	3.36–5.03	0.443	10.6
18.39	28.1	Intact	31	3.24		3.21	2.66–4.37	0.457	14.1
40–79	49.4	Castrate	23	3.68	<.001	3.69	2.86–4.66	0.416	11.3
42–81	55.4	Intact	19	2.98		2.94	2.35–3.90	0.420	14.1

* Standard deviation.
† Coefficient of variation.

1) was somewhat greater at younger than at older ages (0.93 *vs.* 0.70 mg./100 ml.), due to particularly high values in young eunuchs and a more drastic decline in SIP with aging in castrated than in intact males (0.49 *vs.* 0.26 mg./100 ml.).

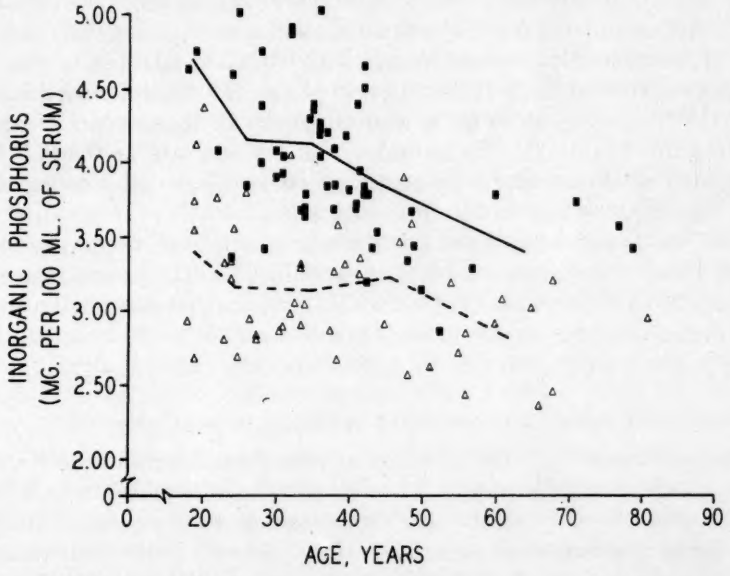

FIG. 1. Higher values for serum inorganic phosphorus in a series of 58 eunuchs (black rectangles) than in a group of 50 intact males (white triangles). The curves (solid line for eunuchs and broken line for intact males) are based on averages for the age groups 18–19, 20–29, 30–39, 40–49 and 50 or more. All subjects were feeble-minded.

TABLE 2. RELATIONSHIP BETWEEN SERUM INORGANIC PHOSPHORUS LEVEL AND AGE AT CASTRATION OR DURATION OF CASTRATE STATE

(Analyses restricted to subjects 20 to 39 years of age at the time SIP was measured; this restriction minimizes complications due to the reduction in SIP with aging—a phenomenon which is less pronounced in the 20–29 and 30–39 year age groups than thereafter.)

Age at castration (yrs.)		No. of subjects	SIP (mg./100 ml.)			Years since castration		No. of subjects	SIP (mg./100 ml.)		
Range	Mean		Mean	Range	Median	Range	Mean		Mean	Range	Median
12–15	13.3	7	4.26	3.69–4.87	4.26	3–11	8.6	10	4.02	3.36–5.02	3.97
16–18	17.0	10	4.08	3.68–4.91	4.06	12–16	14.6	10	4.13	3.63–4.91	3.99
19–29	21.3	13	4.03	3.36–5.02	3.93	19–25	21.4	10	4.14	3.69–4.87	4.19

The depression of SIP concentration by testicular secretions is not established permanently upon sexual maturation but requires the continued presence of these secretions. The level of SIP was elevated in eunuchs, whether they had been orchiectomized after or before sexual maturation. In 30 eunuchs tested when they were 20 to 39 years of age (two decades in which the mean level for SIP remained similar at 4.13 and 4.09 mg. per 100 ml., respectively), values for SIP concentration in subjects who had not been castrated until 19 to 29 years of age did not differ significantly ($P = .15$) from those in subjects who were only 12 to 15 years of age at orchiectomy (Table 2). The immediacy of the increase in the SIP level after removal of testicular secretions is shown by its elevation to a statistically significant extent within fifteen days (1).

The duration of the castrate state, which was three to twenty-five years in the group under study, did not affect values for SIP as analyzed in 30 subjects 20 to 39 years old (Table 2). This confirms and extends the finding of a rather constant degree of elevation of the SIP level observed in the same subjects studied serially for four years after castration (1).

Decline in SIP values with advancing age (feeble-minded subjects)

The decrease in SIP concentration in eunuchs was significant ($P < .001$) over the age span under study (18 to 79 years), as tested by the difference from zero in the slope of the line for linear regression equations fitted to data for each group at all ages. The rate of decrease in SIP concentration was most rapid in the 18–19 and 20–29 year groups, yet values in the 18–19 year group, and in the 20–29 and 30–39 year groups tended to remain within the high range characteristic of childhood. Even in middle or old

age the level of SIP in eunuchs did not fall to that observed in their control intact subjects (Fig. 1).

At or shortly after the age for sexual maturation, values for SIP concentration in the intact feeble-minded were much lower than in the castrate group, in keeping with the depression of the SIP level associated with maturation (2). At 50 or more years of age the intact feeble-minded had a still lower concentration of SIP than at 18 to 39 years of age ($P = .007$), but the decline between these two age groups was much less than in eunuchs (0.43 *vs.* 1.30 mg. per 100 ml.). This observation requires confirmation, since only a small number of old men were studied. Moreover, no significant reduction of SIP concentration with age has been observed (2) in a larger series of intact adult males who were not feeble-minded.

Evidence that the high SIP level in eunuchs is not due to hypofunction of the adrenal cortices

Values for urinary 17-hydroxycorticosteroids—metabolites derived almost solely from adrenocortical secretions and considered to provide a good measure of these secretions (20)—were determined in 51 of the subjects 23 to 39 years old, a span of age when titers of 17-hydroxycorticosteroids did not decline in the subjects under study. There was no significant difference in mean daily titers between the eunuchs and their intact control group of feeble-minded males (8.70 and 8.76 mg., respectively). Furthermore, there was no significant correlation between values for SIP concentration and urinary 17-hydroxycorticosteroids in individual subjects in either the castrate or the intact group.

Measurements were also made of urinary 17-ketosteroids—substances which appear to be derivatives chiefly of adrenocortical secretions (21) and are obviously not metabolites of testicular secretions in castrated men. In 57 feeble-minded males 30 to 49 years old, a range of age during which there was no material decline in 17-ketosteroid values in the subjects under study, mean daily titers of 17-ketosteroids were 13.3 mg. in eunuchs and 12.4 mg. in the intact males. The difference is not significant and, such as it is, is in the direction opposite to that which would account for the high levels of SIP in eunuchs on the basis of depressed adrenocortical function. There was no significant relationship between values for SIP and for urinary 17-ketosteroids in individual subjects in either the castrate or the intact group, in keeping with a similar lack of relationship in intact men of normal mentality (2).

It may be concluded that the elevation of SIP concentration in eunuchs was not due to a decrease in adrenocortical function insofar as can be gauged by urinary titers of 17-hydroxycorticosteroids and 17-ketosteroids.

Lack of relationship between the SIP level and other values under study

In neither the eunuchs nor their intact control group was there a significant correlation between values for SIP concentration and those for other items affected by orchiectomy—sedimentation rate and fragility of erythrocytes; hemoglobin concentration; hematocrit; urinary creatinine excretion; production of sebum; and growth of secondary sex characters such as the beard and axillary hair (4).

Values for SIP were also not significantly correlated with those for serum alkaline phosphatase (which tended to be higher in castrated than in intact males in the 20–29 and 30–39 year age groups; $P = .003$), nor with those for other constituents of serum studied largely for purposes apart from those referred to in this communication, *i.e.*, phospholipids, total cholesterol or alpha- and beta-lipoproteins.

The rate of elongation of nails, which reflects at least some body-wide changes in growth and replication of tissues (22), was not significantly greater in the castrate series than in the intact controls. To the extent that replacement of protoplasm, as in the nail, is related to the secretion and actions of growth hormone, these data support the conclusion reached previously (1) that elevation of the SIP level in subjects with limited or absent gonadal secretions is due to lack of gonadal secretions and not necessarily, as commonly postulated, to a hypothetical increase in titers of growth hormone.

Tendency to low SIP levels in association with feeble-mindedness

In intact males of normal mentality the values for SIP concentration did not differ significantly between institutionalized and the non-institutionalized subjects. Therefore, data for these two mentally-normal groups were combined in the following analyses.

The mean value of 3.6 mg. per 100 ml. in the 110 mentally-normal intact males, 18 to 85 years old, was significantly higher ($P < .001$) than that of 3.1 mg. per 100 ml. in the 50 intact feeble-minded men of comparable age (18 to 81 years). A tendency to low SIP concentration in the feeble-minded is apparent in each of the 5 age groups (18 and 19, 20 to 29, 30 to 39, 40 to 49, and 50 or more years) upon which the curves of mean values in Figure 2 are based.

The low values for SIP concentration in feeble-minded intact adults were not associated with a complete incapacity for normal or supranormal levels of SIP. The SIP level rises rapidly and markedly in the feeble-minded following removal of the testes (1). In the present study, the concentration of SIP in subjects 18 or more years of age was significantly higher ($P < .001$)

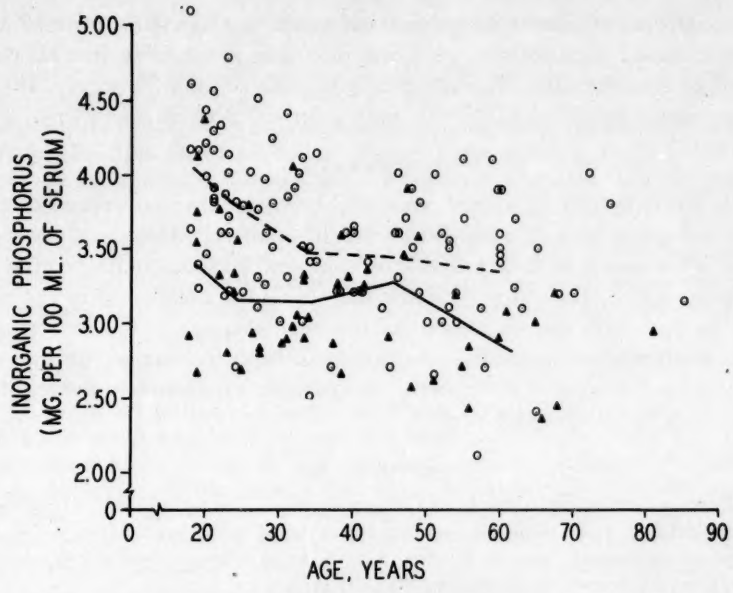

FIG. 2. Lower values for serum inorganic phosphorus in a series of 50 feeble-minded males (black triangles) than in 110 males of normal mentality (circles), as tested in intact subjects. Mean values are shown for the age groups 18–19, 20–29, 30–39, 40–49 and 50 or more.

in feeble-minded eunuchs than in males of normal mentality who were not castrated.

Measurements of urinary 17-ketosteroids and secondary sex characters strongly suggest that the tendency to low values for SIP concentration in feeble-minded intact males is not due to excessive amounts of testicular secretions or to excessive responses by tissues which react to testicular secretions.

Urinary titers of 17-hydroxycorticosteroids and 17-ketosteroids failed to indicate any severe hypofunction of the adrenal cortices which would account for low SIP levels in intact feeble-minded males. Interpretation of these data should be guarded, however. Steroid titers were the only indices of adrenocortical functions under investigation and it was not possible to carry out similar studies in mentally normal males who were housed under conditions that closely approximated those of the feeble-minded.

The low SIP levels in the feeble-minded intact males under study does not account for their feeble-mindedness. After orchiectomy SIP concentration shifted to supranormal levels without concomitant change in intelli-

gence. Moreover, in the feeble-minded subjects the values for SIP were not correlated significantly with the intelligence quotient in either castrated or intact males. The possibility is not excluded, however, that at earlier ages subnormal SIP levels may interfere with development.

COMMENT

The present study furnished considerably more data than reported previously for eunuchs and throughout the lifespan. Control data were provided by a series of intact males of comparable age, mental status and environment. To the writers' knowledge, this is the first report of the tendency to low SIP concentration in the feeble-minded. The observation needs confirmation in studies of subjects of both feeble and normal mentality who live under even more comparable environmental conditions than those in the present study.

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STUDIES OF ADRENAL CORTICOSTEROIDS. I. ESTIMATION OF PLASMA CORTICOSTERONE AND CORTISOL*

ROBERT S. ELY, M.D., EDWIN R. HUGHES, M.D. AND VINCENT C. KELLEY, M.D., PH.D.

The Department of Pediatrics, University of Utah College of Medicine and the State Heart Laboratories, Utah State Department of Health, Salt Lake City, Utah

ABSTRACT

A method is presented consisting of modifications of Sweat's original fluorometric technic for the estimation of corticosterone and cortisol in plasma. These modifications include substitution of ethyl acetate for chloroform as the extracting solvent, replacement of the pentane-alcohol partition step by Florisil chromatography and replacement of the mercury arc lamp for excitation light by a more monochromatic light source. These changes appear to have improved the specificity of the method.

In young adults, plasma concentrations of 3.0 μg. per 100 ml. for corticosterone and 10.9 μg. per 100 ml. for cortisol were obtained. Considerable individual difference was observed when comparing the fluorometric value for cortisol with the Nelson-Samuels value for 17-OHCS. General problems remain concerning the identification and quantitation of corticosterone, as indicated by the disparity among values obtained for corticosterone in peripheral human plasma by the various methods used.

THERE now are available several methods for the estimation of adrenocortical steroids in plasma and urine. To a varying degree they have in common a lack of specificity. With the small amounts of steroid present in plasma, much depends upon purification and resolution of the steroids involved; consequently the interpretation of results is subject to reservations.

The role of corticosterone, a major corticosteroid in most species, has not been subjected to intense investigation chiefly because of difficulty in methodology. Sweat (1, 2) introduced a technic for the estimation of corticosterone (compound B or Δ^4-pregnene-11β,21-diol-3,20-dione) and cortisol (compound F or Δ^4-pregnene-11β,17α,21-triol-3,20,dione) which consisted of chloroform extraction, pentane-70% methanol partition, chromatography with a silica-gel micro-column, and measurement of the eluted steroids on the basis of their sulfuric-acid fluorescence. The specificity of this technic is dependent upon purification of the plasma sample and the fluor-

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escent characteristics of the resolved corticosteroids. During the course of investigations in this laboratory, several modifications of Sweat's original fluorometric method were made which in our hands permit more satisfactory estimation of corticosterone and cortisol in small volumes of plasma. In the present report this modified method is described; details of Sweat's original method which have remained unchanged are presented only by reference whenever this is possible without destroying continuity.

MATERIALS AND METHODS

A. *Apparatus*

1. *Micro-column.* The 126-mm. chromatographic column described by Sweat (1) is employed; however, the ground-glass joint between the column and the water jacket, and the stopcock are omitted. This column is used for both Florisil and silica-gel chromatography.

2. *Instruments.* A modified Farrand (Model 1040) fluorometer is used. The mercury lamp and primary filter system for producing the excitation light are removed; instead, the primary focusing lens of the fluorometer is connected by a non-absorbing cylinder to a model B Beckman spectrophotometer with its tungsten light source. Readings are taken using a wave length of 480 mμ for the excitation light. The secondary filters used are Wratten gelatin #7 and #16, transmitting fluorescent light in the range of 520 to 580 mμ, with a principal transmission band at 540 mμ.

B. *Reagents*

1. *Chloroform.* Analytical reagent grade (Mallinckrodt), supplied in glass carboys. This is distilled over 5 Gm. of anhydrous potassium carbonate, the first and last 10 per cent of the distillate being discarded. Five to 10 liters of chloroform are distilled[1] at one time and stored in the cold. There is little change in polarity or background fluorescence up to twenty or thirty days.

2. *Methanol.* Absolute, analytical reagent grade. This is distilled[1] over 2–5 Gm. of 2,4-dinitrophenylhydrazine, the first and last 10 per cent of the distillate being discarded.

3–5. *Ethyl acetate, diethyl ether and acetone.* Analytic reagent grade, in glass containers. These also are distilled[1] before using.

6. *Sulfuric acid.* C.P. reagent grade, concentrated (Dupont).

7. *Powdered glass.* Sizes 20–30, 30–40, 40–50 mesh (1) (Corning).

8. *Silica gel.* Through 200 mesh (Davison Chemical Corporation). The gel is washed and activated as described by Sweat (1).

9. *Florisil.* Size 60–100 mesh (The Floridin Corporation). It is prepared and activated as described by Nelson and Samuels (3).

C. *Procedure*

The plasma sample is extracted with ethyl acetate. This extract is evaporated to dryness, redissolved in chloroform, and chromatographed on a Florisil micro-column. The proper eluate is collected, evaporated, re-dissolved in chloroform, and chromatographed on a silica-gel micro-column. Each of the resulting eluates is evaporated to dry-

[1] Distilled in all-glass, water-free distillation apparatus fitted with a fractionating column of from 1–1.5 theoretical plates.

ness and re-dissolved in methanol. Concentrated sulfuric acid is added to the flask and the resulting fluorescence is compared with that for standard steroid.

1. *The plasma sample.* Blood is obtained in heparinized syringes by venipuncture, and the plasma separated as soon as possible. The plasma samples are stored in a frozen state until determinations are performed.

2. *Extraction.* A 5-ml. sample of plasma in a 20-ml. test tube is extracted three times with ethyl acetate (10 ml., 10 ml. and 5 ml., respectively). The extraction is performed by shaking the mixture by hand for thirty seconds followed by centrifuging for five minutes at 3000 rpm. Each ethyl-acetate extract is decanted into a 25-ml. round-bottom flask, the transfer of any plasma droplets being carefully avoided. The combined extract is evaporated to dryness by vacuum suction at 50° C. on an agitating water bath. All subsequent evaporations are conducted in the same manner.

3. *Florisil column chromatography.* The Florisil micro-column is prepared as follows: The micro-column and its water jacket are rinsed on all surfaces with chloroform followed by absolute methanol. A 5-mm. layer of powdered glass is introduced into the micro-column. This is added as equal quantities of 20–30 mesh, 30–40 mesh and 40–50 mesh, successively, each being washed into place with chloroform. The column is allowed to dry; then Florisil (1.5 Gm.) is added and packed by gentle tapping on the outside of the column with a rubber-sleeved glass rod (care being exerted to pack all columns similarly); finally, 20–30 mesh powdered glass (2–3 mm.) is placed on top of the Florisil.

The dried ethyl-acetate extract is redissolved in 0.5 ml. of chloroform; at least fifteen minutes of contact is allowed. A 5-ml. portion of chloroform is introduced into the Florisil column. When the descending upper surface of chloroform approaches the upper surface of the Florisil,[2] the dissolved extract is transferred cautiously by pipette[3] to the column.[4] The sample flask is washed twice with 0.5 ml. of chloroform, each wash being transferred to the column in a similar manner. When the descending upper level of the final wash approaches the upper surface of the Florisil, the column is developed by adding 10 ml. of chloroform. The corticosteroids then are eluted fractionally by 10 ml. of 2 per cent methanol-chloroform followed by 20 ml. of 25 per cent methanol-chloroform. The more polar steroids, including corticosterone and cortisol, are eluted in this final fraction which is collected in a 25-ml. round-bottom flask and evaporated to dryness. This residue is rechromatographed on the silica-gel micro-column.

4. *Silica-gel column chromatography.* The silica-gel micro-column is prepared in the following manner: The initial steps in preparation of the column are as described previously. After the ground glass is in place, a slurry of 0.5 Gm. of silica gel in 5 ml. of chloroform is added. Additional small volumes of chloroform are used to rinse the beaker and the bowl of the column. As soon as the descending upper level of chloroform approaches the upper surface of the silica gel, a 10-ml. portion of 1:1 ethyl ether-acetone is added. This is followed by two 10-ml. volumes of chloroform. These washes are used to remove traces of moisture and to clean the column of extraneous fluorescing material. They are listed in Table 1 as fractions A, B and C.

During this time the dried 25 per cent methanol-chloroform eluate from the Florisil column has been redissolved in 0.5 ml. of chloroform (by carefully swirling the chloro-

[2] Care should be taken that no portion of the Florisil becomes exposed to air from this point on, and similar precautions must be taken during silica-gel chromatography.

[3] Plain Pyrex pipette, 0.7-ml. capacity, Rheberg tip, fitted with rubber bulb (Bicknell and Associates).

[4] Care must be exercised in the transfer so that none of the residue is lost and the adsorbent is not disturbed.

form within the round-bottom flask so that the entire bowl surface has been wetted) and allowed to stand fifteen minutes. The sample then is transferred to the silica-gel column (as described for Florisil column chromatography). The volumes and types of solutions used subsequently for chromatography are shown in Table 1. The eluates are collected individually in 25-ml. round-bottom flasks and evaporated to dryness.

5. *Development of fluorescence.* A wave length of 480 mμ is selected on the Beckman Model B spectrophotometer. The Farrand fluorometer is adjusted for use as described by Sweat (2). The fluorescence of a known amount of standard steroid, added directly into the cuvette, is used as a reference for all samples. The sensitivity of the fluorometer is set so that the fluorescence of 1 microgram of standard steroid reads 100 on the galvanometer. The standards[5] are prepared in duplicate as follows: Known amounts of standard corticosterone (0.25, 0.5 and 1.0 μg., respectively) are introduced[6] into 25-ml. round-bottom flasks and 200 μl. of methanol added to each. The flasks are placed in a Kahn shaker[7] and agitated vigorously for one minute. Then 1.0 ml. of concentrated sulfuric acid is added to each flask and the flask is shaken for three minutes. The contents of the flasks are poured into matched 10×75 mm. Pyrex test tubes, and the fluorescence determined. Cortisol standards are prepared in the same manner except that only 100-μl. volumes of methanol are added.[8] Duplicate blanks are prepared in the same manner, 200 μl. of methanol and 1.0 ml. of sulfuric acid being used for the corticosterone blank, and 100 μl. of methanol and 1.0 ml. of sulfuric acid for the cortisol blank. For chromatographed samples, the eluates through fraction 5 are read against the corticosterone standard and are prepared by adding 200 μl. of methanol and 1.0 ml. of sulfuric acid to the flask (carefully wetting the entire inner surface of the flask with the methanol). Fractions 6 and 7 are read against the cortisol standard and are prepared by adding 100 μl. of methanol and 1.0 ml. of sulfuric acid.

6. *Analysis of fluorescence.* The standard curve for the day is determined by plotting the fluorescence reading versus concentration for each standard steroid. For both corticosterone and cortisol in amounts from 0.1 to 1.0 μg. this is a straight-line relationship. For the chromatographed sample (plasma or standard steroid), the concentration of corticosterone is estimated by using the standard curve for corticosterone and the fluorometric reading for fraction 4. Similarly, the value for cortisol is estimated by using the standard curve for cortisol and the fluorometric reading for fraction 6.

EXPERIMENTAL

Fluorescence of eluted fractions from chromatography of solvent blanks

Table 1 shows the composition and volume of each eluent used in silica-gel column chromatography as employed in this study. Initially, the column is cleaned with 10 ml. of 1:1 ether-acetone (fraction A) followed by two washings with 10 ml. of chloroform (fractions B and C). At this point

[5] Standard steroids used in these studies were supplied through the courtesy of Dr. C. J. O'Donovan, The Upjohn Company, Kalamazoo, Mich.

[6] With a 100-μl. transfer pipette, Kirk type (Micro-chemical Specialties Co.).

[7] The flasks are held secure in the shaker by placing them in the cardboard "flats and fillers" used for crating eggs.

[8] At an excitation wave length of 480 mμ a more intense fluorescence for corticosterone is achieved by a 2:10 methanol-H_2SO_4 ratio and for cortisol by a 1:10 ratio. Bondy (4), using the mercury arc lamp, recommends a 3:7 methanol-H_2SO_4 ratio for corticosterone.

TABLE 1. CHROMATOGRAPHY OF STANDARD CORTICOSTERONE AND CORTISOL

	Eluent		Elution fraction	Fluorometric reading*				
				1	2	3	4	5
	Vol. (ml.)	Composition		Solvent blank†	Standard,‡ 1 μg.B	Standard,‡ 1 μg.F	Standard 1 μg.B and 1 μg.F	
							In alcohol‡	In saline†
Cleaning of column	10	1:1 Ether-Acetone	A	25	10	12	15	18
	10	Chloroform	B	3	7	8	10	10
	10	Chloroform	C	1	4	5	3	4
Addition of sample								
Development and elution of column	20	Chloroform	0	12	9	10	10	12
	5	Chloroform	1	0	2	3	4	3
	5	0.5% Methanol-Chloroform	2	0	2	1	2	1
	5	1.0% Methanol-Chloroform	3	0	3	0	4	5
	20	1.0% Methanol-Chloroform	4	1	86	3	92	65
	5	1.0% Methanol-Chloroform	5	1	4	7	5	8
	15	5.0% Methanol-Chloroform	6	4	6	93	89	73
	10	5.0% Methanol-Chloroform	7	0	1	8	4	7
Steroid recovered (μg.)								
Corticosterone					0.86		0.92	0.65
Cortisol						0.93	0.89	0.73

* Fluorometric value of 1 μg. of corticosterone or cortisol in cuvette = 100 galvanometric deflection units.
† Carried through entire procedure.
‡ Silica-gel chromatography only.

the sample is added to the column. Development and fractional elution then is carried out with eluents, as indicated in the table (fractions 0 to 7, inclusive).

As shown in the table (column 1), considerable background fluorescent material appears in the initial cleaning of the column (fractions A, B and C), with only a small amount appearing in fraction C. This reappears transiently to a lesser extent in fraction 0, following addition of the "sample."[9] However, fractions 1 through 7 have slight background fluorescence.

Fluorescence of eluted fractions from chromatography of standard steroids

During chromatography of the standard steroids (Table 1), just as with the solvent blanks, a considerable amount of background fluorescent material appears in the initial cleaning of the column and reappears transiently upon addition of the sample, but again fractions 1 through 7 have only slight background fluorescence.

[9] The "sample" consists of the dried residue of all solvents used through Florisil chromatography.

Essentially all of the corticosterone appears in fraction 4, only small amounts appearing in fractions 3 and 5. Practically all of the cortisol appears in fraction 6. This is shown in the table by illustrative elution patterns obtained upon chromatography of 1 μg. of corticosterone (column 2), 1 μg. of cortisol (column 3), and a mixture of 1 μg. of corticosterone and 1 μg. of cortisol (column 4). As can be seen, the resolution is such that the presence of one steroid has no appreciable influence upon the fluorescent activity of the other.

The recovery of standard steroid from silica-gel chromatography is about 90 per cent. However, a lesser recovery is obtained when standard steroid is carried through all steps of the method, *i.e.*, initial extractions and Florisil chromatography in addition to the final silica-gel chromatography. This is shown in column 5 of Table 1; recovery from saline was 65 per cent for corticosterone and 73 per cent for cortisol. In general, the recoveries in similar experiments have ranged from 60 to 80 per cent.

Fluorescence of elution fractions from chromatography of human plasma

Illustrative elution patterns obtained from chromatography of 5-ml. aliquots of human plasma are shown in Table 2. Included are the patterns from a plasma sample (Subject J.B.), to one aliquot of which 1 μg. of corticosterone and 1 μg. of cortisol had been added, and those from plasma samples (Subject R.K.) obtained before and one hour after the intravenous administration of ACTH (0.2 I.U./Kg.). Generally, plasma produces a background fluorescence in fraction 0 (the first elution fraction after addition of the sample) which is three to four times that for standard corticosterone and cortisol. Part of this fluorescence presumably is attributable to steroid (1), but not to corticosterone or cortisol.

In addition to the fluorescent activity of fraction 0, two peaks are found in plasma from the fasting human subject. A smaller peak occurs in fraction 4, coinciding with that for standard corticosterone. In the 2 fasting plasma samples shown in Table 2 the fluorescence of fraction 4 was equivalent to that of 0.17 μg. and 0.19 μg. of corticosterone per sample, respectively. A larger peak occurs in fraction 6, coinciding with that for standard cortisol; the fluorescence of this fraction in the 2 samples was equivalent to that of 0.45 μg. and 0.72 μg. of cortisol, respectively.

Recovery of standard corticosterone and cortisol from plasma is somewhat less than that from saline. Whereas recovery from saline ranged from 60–80 per cent, that from plasma ranged from 50–70 per cent. From the plasma of Subject J.B. (Table 2), 61 per cent of the added corticosterone and 64 per cent of the added cortisol were recovered. Resolution was satisfactory, as there was little change in the fluorescence of fractions other than Nos. 4 and 6 following the addition of the standard steroids.

TABLE 2. CHROMATOGRAPHY OF CORTICOSTERONE AND CORTISOL FROM HUMAN PLASMA*

Elution fraction†	Fluorometric reading				
	Standard, 1 μg. B+ 1 μg. F	Subject J.B.		Subject R.K.‡	
		Plasma§	Plasma (1 μg. B+ 1 μg. F added)	Plasma before ACTH	Plasma after ACTH
A	11	20	18	11	16
B	3	10	14	8	6
C	0	4	8	2	4
Addition of sample					
0	7	30	24	30	23
1	0	5	10	3	5
2	1	7	2	3	2
3	4	10	12	4	9
4	87	17	78	19	48
5	6	4	9	7	10
6	90	45	109	72	138
7	4	8	12	4	10
μg. Steroid in plasma sample:					
Corticosterone		0.17	0.78	0.19	0.48
Cortisol		0.45	1.09	0.72	1.38

* All plasma samples represent 5-ml. volumes.

† See Table 1 for volume and comosition of each elution fraction.

‡ Fasting sample obtained; then 10 I.U. (0.2 I.U./Kg.) of ACTH administered intravenously; second sample obtained one hour later.

§ The 17-hydroxycorticosteroid content (Nelson and Samuels method (3, 5)) of an aliquot of this plasma was 0.5 μg./5.0 ml.

In plasma obtained following administration of ACTH *in vivo*, there also is an increase in fluorescence of both the corticosterone and cortisol elution fractions; however, no appreciable change occurs in fluorescence of the other fractions. In the example shown in Table 2 (Subject R.K.), the increase in fluorescence of elution fraction 4 was equivalent to an elevation of corticosterone concentration from 0.19 μg. to 0.48 μg. per 5 ml. of plasma; that of fraction 6 was equivalent to an elevation of cortisol concentration from 0.72 μg. to 1.38 μg. per 5 ml. of plasma.

Resolution of the 2 steroids remains good even when a relatively large amount of one is present in plasma, *i.e.*, following intravenous administra-

tion of corticosterone or cortisol. In Table 3 and Figure 1 are shown data illustrating the fluorescence of human plasma samples obtained serially following infusion of corticosterone or cortisol (2 mg./Kg.). In each case the thirty-minute plasma sample showed a marked increase in fluorescence for the elution fraction corresponding to the steroid administered. Thereafter, this fluorescence returned progressively toward normal. In fact, three hours after infusion of corticosterone, the fluorescence fell below control values in both fractions 4 and 6, suggesting an inhibitory effect of the exogenous steroid upon endogenous secretion of both corticosterone and cortisol. Despite the greatly increased fluorescence of fractions 4 and 6 (corresponding to corticosterone and cortisol, respectively), there was only a small increase in the fluorescence of other fractions. Following infusion of corticosterone, there was some increase in fluorescence in fractions 5, 6 and 7 of the thirty-minute and sixty-minute plasma samples, but none thereafter. Also, there was a slight increase in fluorescence in fraction 7 after the intravenous administration of exogenous cortisol. Usually the total increase in fluorescence of adjacent elution fractions is no greater than 10–20 per cent of the increase for the specific elution fraction

TABLE 3. CHROMATOGRAPHY OF HUMAN PLASMA* FOLLOWING INTRAVENOUS ADMINISTRATION OF CORTICOSTERONE OR CORTISOL†

Elution fraction‡	Fluorometric reading											
	Standard, 1 μg. F + 1 μg. B	Time (min.) after infusion of corticosterone						Time (min.) after infusion of cortisol					
		0	30	60	90	120	180	0	30	60	90	120	180
A	9	7	15	12	8	11	10	8	12	8	13	11	14
B	3	2	5	4	3	3	2	2	5	2	3	2	4
C	1	0	1	0	2	1	0	1	2	0	1	2	1
Addition of sample													
0	6	20	19	26	22	18	22	3	12	16	11	14	10
1	0	4	4	2	5	3	4	0	2	5	1	4	2
2	1	2	5	3	2	1	3	1	1	2	3	3	1
3	3	9	12	9	5	9	10	1	2	1	1	0	1
4	85	25	256	104	52	31	15	13	16	9	15	9	16
5	9	5	23	10	5	6	3	0	6	4	3	3	1
6	100	72	91	51	45	38	34	27	370	297	251	209	124
7	2	2	9	8	2	2	2	1	21	13	13	11	8
μg. Steroid recovered													
Corticosterone	0.85	.25	2.56	1.04	.52	.31	.15	.13	.16	.09	.15	.09	.16
Cortisol	1.00	.72	.91	.51	.45	.38	.34	.27	3.70	2.97	2.51	2.09	1.24
Concentration (μg./100 ml.)													
Corticosterone		5	51	21	10	6	3	3	3	2	3	2	3
Cortisol		14	18	10	9	8	7	5	74	59	50	42	25

* Each sample represents a 5-ml. volume.

† Free steroid alcohol infused in a concentration of 0.5 mg./ml; dose administered was 2 mg./Kg. The alcoholic solutions of cortisol and corticosterone were furnished through the courtesy of Dr. C. J. O'Donovan, The Upjohn Co., Kalamazoo, Mich.

‡ See Table 1 for volume and composition of each elution fraction.

(No. 4 or 6) corresponding to the steroid administered. When the logarithms of the values obtained after infusion were plotted against time a straight line resulted in each case (Fig. 1), indicating a first-order reaction for each steroid, with a half-life of thirty minutes for corticosterone and one of ninety-seven minutes for cortisol.

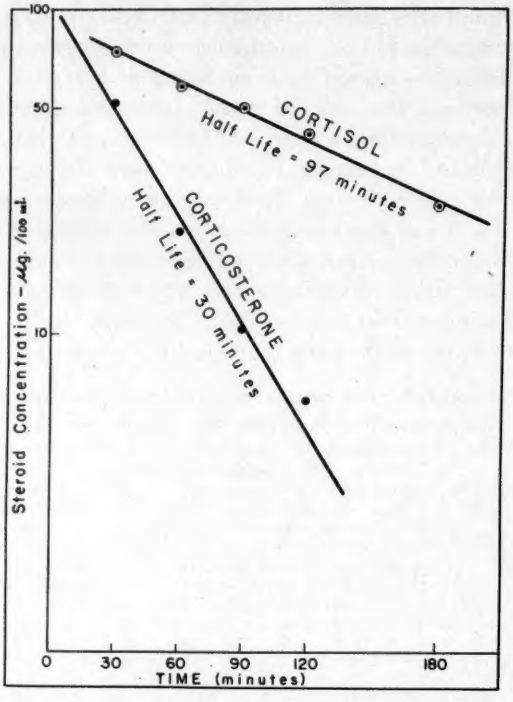

FIG. 1. Rate of disappearance of administered steroid.

Calculation of steroid concentrations

Theoretically, the equation used in calculating the concentration of corticosteroid in a plasma sample should include correction factors for the recovery of standard steroid and for background fluorescence in plasma. In an attempt to determine the most satisfactory method for calculating steroid concentration from the fluorometric readings, aliquots of pooled plasma[10] were processed on different days, and recovery of standard hormone was determined each day. Four aliquots were processed on 9 different days, recovery being compared with that of standard steroid. In addition,

[10] Guinea-pig plasma was used.

aliquots were processed on 4 days by the method of Nelson and Samuels (3, 5).

The results of these analyses are shown in Table 4. When the total fluorometric reading for elution fractions 3 to 5, *corrected* for background activity and for per cent recovery of standard corticosterone, was compared with that for fraction 4 alone, *uncorrected*, the results were in good agreement. Therefore, the latter method of calculation was used. For cortisol, the uncorrected fluorometric reading for fraction 6 was lower than the total reading for fractions 6 and 7, corrected for background activity and per cent recovery of standard. The uncorrected value seemed preferable, since it correlated well with the mean 17-hydroxycorticosteroid (17-OHCS) value as determined by the Nelson and Samuels technic.

TABLE 4. ANALYSIS OF FLUOROMETRIC READING

Type of calculation	No. of aliquots	Fluorometric reading	
		Cortisol	Corticosterone
1. Correcting for background, plus % recovery of standard steroid.	35	55 ±3.1	17 ±1.8
2. Using fluorometric readings in fraction 4 or 6, without correction.	35	41 ±2.7	16 ±1.6
3. Value by Nelson and Samuels method*	15	43 ±3.0	

* Content of 17-hydroxycorticosteroid in μg., converted into the comparable fluorometric reading for standard cortisol.

Steroid concentrations in human plasma

Plasma concentrations of corticosterone and cortisol were determined by the fluorometric method in 20 normal young adults, as shown in Table 5. The mean corticosterone value was 3.0 ±0.20 μg. per 100 ml. and that for cortisol was 10.9 ±0.55 μg. per 100 ml. On an aliquot of each plasma sample, 17-hydroxycorticosteroid concentrations were also determined by the method of Nelson and Samuels (3, 5) in order to compare them with the concentrations of cortisol determined by the fluorometric method. The mean concentrations (10.9 μg. per 100 ml. for cortisol and 9.8 μg. per 100 ml. for 17-OHCS) were in good agreement, the difference between the means being 1.1 ±1.05 μg. per 100 ml. However, when comparison was made between these two values in each individual plasma sample the correlation was very poor: $r = +0.22$. These findings indicate either that there is a lack of precision in one or both of the methods or that certain steroids are present which are measured by one method but not by the other—at least in some fasting plasma samples.

TABLE 5. PLASMA CORTICOSTERONE, CORTISOL AND 17-HYDROXYCORTICOSTEROID LEVELS IN NORMAL YOUNG ADULTS

No.	Subject	Fluorometric method		Nelson and Samuels method
		B (μg./100 ml.)	F (μg./100 ml.)	17-OHCS (μg./100 ml.)
1	D.T.	2.2	8.6	8.1
2	M.N.	2.2	9.2	9.1
3	M.T.	2.8	10.6	12.7
4	E.H.	3.2	10.0	15.0
5	R.H.	2.6	9.0	7.4
6	K.A.	3.8	13.6	14.0
7	O.R.	2.0	11.0	9.3
8	T.H.	2.6	11.0	11.9
9	E.C.	2.8	10.0	11.0
10	C.D.	3.2	12.2	16.1
11	M.M.	3.2	10.0	6.7
12	R.B.	5.2	13.4	1.7
13	M.R.	4.0	16.4	7.9
14	C.W.	3.4	5.0	4.4
15	V.K.	3.6	12.4	18.0
16	A.D.	4.4	13.6	7.9
17	M.C.	2.2	11.4	8.6
18	R.E.	2.8	11.6	11.1
19	T.G.	2.4	10.6	7.5
20	J.G.	2.2	7.8	7.8
Mean		3.0	10.9	9.8
SEM		±0.20	±0.55	±0.89

Difference between the means (F—17-OHCS) 1.1
SEM ±1.05
Correlation coefficient, $r_{F\text{-}17OHCS} = +0.22$

In these young adults the ratio of plasma cortisol to corticosterone was about 3.5 to 1. When the magnitude of response to administered ACTH for the 2 steroids was evaluated, a similar ratio was seen. These data are shown in Table 6 for a group of 21 normal children ranging in age from 10 to 17 years.[11] The means of the control corticosterone and cortisol plasma concentrations were 3.1 ±0.30 μg. and 11.4 ±1.07 μg. per 100 ml., respectively. One hour after ACTH was infused intravenously (0.2 I.U./Kg.) the second sample of plasma was obtained. The mean *increase* over the fasting plasma value for corticosterone was only 2.2 μg. per 100 ml.,

[11] Age relationships will be discussed in another publication.

TABLE 6. PLASMA CORTICOSTERONE AND CORTISOL LEVELS (FLUOROMETRIC METHOD) BEFORE AND AFTER ACTH*

Subject†	Corticosterone (μg./100 ml.)		Cortisol (μg./100 ml.)	
	Before	Increase after ACTH	Before	Increase after ACTH
S.G.	5.9	−0.5	21.2	−0.3
R.C.	4.4	2.7	8.2	10.4
C.E.	4.8	3.4	9.5	16.0
S.Y.	4.2	4.9	9.2	4.5
R.R.	2.2	6.5	15.1	8.3
J.N.	3.8	2.3	11.5	9.8
R.C.	2.6	5.4	3.4	12.4
D.J.	2.8	0.5	10.0	1.1
E.G.	4.0	0.2	16.6	8.1
W.A.	3.9	0.3	6.7	2.5
J.I.	4.4	0.1	16.5	6.1
J.H.	2.4	1.5	19.6	1.1
B.E.	1.2	0.6	9.8	8.5
B.H.	4.4	−1.4	18.7	0.8
T.M.	1.5	3.2	12.7	8.7
D.L.	1.3	4.3	4.3	21.0
L.H.	0.9	1.2	11.1	8.9
J.O.	1.9	2.2	7.9	13.5
K.H.	2.6	2.0	8.9	12.4
S.H.	3.1	1.0	10.3	8.1
K.H.	2.4	4.9	7.7	16.3
No. of subjects	21	21	21	21
Mean	3.1	2.2	11.4	8.5
SEM	±0.30	±0.47	±1.07	±1.23

* ACTH administered intravenously, 0.2 I.U /Kg.; second sample obtained one hour after infusion.

† Subjects were normal children ranging in age from 10 to 17 years.

compared with an increase of 8.5 μg. per 100 ml. for cortisol. The cortisol and 17-OHCS values in fasting plasma corresponded fairly well on the whole but, for reasons not clear, the observed elevations in cortisol concentrations in response to ACTH were considerably lower than the 17-OHCS elevations reported previously (6).

DISCUSSION

The fluorometric method for determination of plasma corticosteroids has value, in that it allows the estimation of corticosterone concentration in a relatively small volume of plasma and allows simultaneous estimation of

both corticosterone and cortisol. These are the two principal adrenocortical hormones in most species studied thus far. The advantages of using this method for estimating cortisol are chiefly theoretical, since simpler technics are available using the Porter-Silber reaction (7). The fluorometric method measures cortisol independently of steroids with a similar side-chain (compounds S and E). However, since these other steroids generally represent only a small fraction of the 17,21-dihydroxy-20-ketosteroids present, there is little advantage derived from independent measurement of cortisol.

As shown by the data in Table 5, the mean values obtained for 17-OHCS by the Nelson and Samuels method and for cortisol by the fluorometric technic in a group of normal young adults were in good agreement, but the correlation was poor when individual values were compared ($r = +0.22$). This may be attributable entirely to inaccuracies in one or both methods. However, it is possible that other factors are important. The following observations made in our laboratory lend credence to this latter possibility: 1. Agreement between results of the two methods is better for solutions of standard steroids than for plasma samples; 2. Agreement is better for plasma samples from normal individuals than for those from patients with rheumatic fever, asthma, and certain other disorders; 3. Agreement is better for plasma samples from normal human subjects than for those from certain other species (*e.g.*, rat, rabbit).

Theoretically, the values for 17-OHCS should be higher than those for cortisol if appreciable quantities of 17,21-dihydroxy-20-ketosteroids other than cortisol be present. This might explain our finding in man that following administration of ACTH the increase in 17-OHCS concentration is greater than the increase in cortisol concentration. We have also observed this difference in rats—secretors of corticosterone primarily—in which stimulation with ACTH causes a significant increase in 17-OHCS concentration without a corresponding increase in cortisol concentration.

The converse—the occurrence of higher values for cortisol than for 17-OHCS—cannot be explained this way. Other than errors in methods, one explanation for discrepancies of this type might be the presence of some substance which appears with cortisol in elution fraction 6, and which fluoresces. Another possibility is that some steroid may be present which interferes with the measurement of 17-OHCS by the Nelson and Samuels technic. Although there is no evidence to implicate a compound in human plasma as being important in this regard, it has been found that 17-hydroxyprogesterone, an intermediary compound in the process of steriodogenesis, will cause spuriously low results for 17-OHCS determinations with cortisol standards.

The development of a satisfactory method for determining corticoster-

one has presented difficulties. Concentrations of this steroid of the peripheral circulation are low, even in those species in which corticosterone is the predominant corticosteroid. The technics generally have lacked the desired sensitivity or specificity. As a result, relatively few studies concerning corticosterone have been reported. Sweat introduced for the first time a method for estimating corticosterone in small amounts of biologic fluids, thus facilitating studies in man and small animals (1, 2, 8). However, although the method has good sensitivity and permits satisfactory resolution of corticosterone and cortisol, the problem of specificity has not been resolved satisfactorily. Other steroidal material will fluoresce in combination with sulfuric acid. This and other extraneous fluorescence become relatively more significant with decreasing amounts of corticosterone in the sample. Thus, the problem of specificity is the major limitation of the fluorometric method.

Therefore, purification of the sample is vitally important, both to remove other steroidal fluorescence and to minimize the influence of extraneous material. The major changes in *purification* in the method presented here, compared with Sweat's original method, are: the use of ethyl acetate rather than chloroform in plasma extractions, and substitution of Florisil column chromatography for the pentane-alcohol partition step. From Burstein's experience with partition coefficients of $C_{21}O_6$ steroids (9) it might be expected that ethyl acetate would extract corticosterone, as well as cortisol, more efficiently than chloroform. Moreover, in our experience, ethyl acetate extracts of plasma contain less "background" fluorescence than do chloroform extracts; this is in agreement with Meyer's report (10) of 50 per cent less background fluorescence with ethyl acetate than with ether-chloroform.

The other major modification which was made in the purification procedure—substitution of Florisil column chromatography for the pentane-alcohol partition step—probably is of more importance in purification of the sample. This substitution was instituted upon discovery that rat plasma and guinea pig plasma contained material which fluoresced excessively when the pentane-70% methanol partition was used. Replacement of the latter with Florisil chromatography resulted in a significant reduction of fluorescence without corresponding loss of corticosterone or cortisol. Table 7 illustrates the fluorescence obtained from aliquots of plasma, comparing the two purification steps. Standard steroid recoveries were not influenced by the purification process. However, the general fluorescence from rat and guinea pig plasma was reduced considerably when Florisil chromatography was used; as a result the corticosterone and cortisol fractions emerged more clearly. Although human plasma exhibited this reduction in general fluorescence, it was to a lesser extent. In the examples shown, the

TABLE 7. FLUORESCENCE OF ELUTION FRACTIONS: COMPARISON OF PENTANE-70% METHANOL PARTITION WITH FLORISIL COLUMN CHROMATOGRAPHY FOR PURIFICATION OF THE PLASMA EXTRACT*

Elution fraction†	Fluorometric reading							
	Standard, 1 μg. F+1 μg. B		Rat		Guinea pig		Human	
	Pentane partition‡	Florisil§	Pentane partition	Florisil	Pentane partition	Florisil	Pentane partition	Florisil
Addition of sample								
0	11	9	25	8	12	10	15	10
1	2	1	3	3	5	2	13	5
2	1	2	8	2	15	3	10	4
3	7	4	11	3	43	14	19	6
4	65	70	61	41	90	27	43	22
5	7	8	8	6	30	8	5	4
6	69	74	103	24	211	153	60	73
7	6	4	83	5	27	9	11	7

* Each sample represents a 5-ml. volume.

† See Table 1 for volume and composition of each elution fraction.

‡ Carried through entire procedure, using a pentane-70% methanol partition step in place of Florisil chromatography.

§ Carried through entire procedure, using Florisil chromatography.

reduction in over-all fluorescence which resulted from using Florisil was roughly 70 per cent for rat plasma, 50 per cent for guinea pig plasma and 25 per cent for human plasma.

Under the conditions used, the maximal fluorescence for corticosterone and for cortisol resulted with an excitation light at 480 mμ; this is in close agreement with previous reports (2, 11). Therefore, the mercury arc lamp, gelatin filter system was replaced by the more monochromatic light source of the Beckman Model B spectrophotometer for greater specificity of the excitation light. In this way extraneous fluorescence—or that from other steroidal material, except for those steroids also absorbing maximally at this wave length—was diminished. The tungsten lamp of the spectrophotometer emits a less intense light than the mercury arc lamp, but the sensitivity of the fluorometer is sufficient to compensate for this.

The extent to which other steroidal material also may be present in the final eluates is unknown. Those steroids studied by Sweat (2) which exhibited sulfuric-acid fluorescence comparable to that of corticosterone or

cortisol are: 1) the 11α epimers of corticosterone and cortisol, 2) tetrol F (Reichstein's E: Δ^4-pregnene-11β,17α,20,21-tetrol-3-one), and 3) estradiol. Goldzieher (11) also found a high sulfuric-acid fluorescence for androsterone, etiocholanolone, isoandrosterone, estrone, estradiol and estriol, but

TABLE 8. COMPARISON OF PLASMA CORTICOSTERONE LEVELS FOUND BY VARIOUS INVESTIGATORS USING VARIOUS METHODS

Author	Method	No. of subjects*	Plasma corticosterone (μg./100 ml.)
1. Bush & Sandberg (13)	Column chromatography, then paper chromatography	4	<2
2. Morris & Williams (14)	Partition chromatography, then polarography of the Girard hydrazones	13	7.8
3. Morris & Williams (15)	Partition chromatography, then ultraviolet (UV) absorption and diphenyl tetrazolium (B.T.) reduction	6	11.0 (UV) 12.6 (B.T.)
4. Sweat (8)	Silica-gel micro-column chromatography, then sulfuric acid fluorescence	21	4.3
5. Starlinger & Tamm (16)	Modied Sweat method	15	1.6
6. Takeda (12)	Silica-gel micro-column chromatography, then phosphoric-acid fluorescence	17	6.1
7. Bondy & Upon (4)	Paper chromatography, then sulfuric-acid fluorescence with isotope dilution for recovery correction	29	1.3
8. Peterson (17)	Chromatography, then sulfuric-acid fluorescence with isotope dilution for recovery correction	20	1.1
9. Weischelbaum (18)	Column chromatography, then Madêr-Buck reaction (B.T. reduction) minus Porter-Silber value	29	8.9 (as 17-desoxycorticosteroids)
10. Lewis (19)	Reverse-phase paper chromatography	30	1.2
11. Present report	Florisil micro-column chromatography, then silica-gel micro-column chromatography and sulfuric-acid fluorescence	20	3.0

* Subjects represent young adults in all instances in which age was mentioned in the original publication.

the experimental conditions were considerably different. Takeda (12), using paper chromatography, found evidence of 2 substances other than corticosterone in the corticosterone elution fraction resulting from silica-gel column chromatography of human plasma; the question of the nature and quantity of these was not clarified. Although most of those steroids known to have significant sulfuric-acid fluorescence will be separated from cortisol and corticosterone by the method employed here, there still may be some steroidal fluorescence other than that due to corticosterone in the elution fractions containing the latter.

The difficulty in accurately measuring corticosterone is emphasized by the disparity among results for human plasma concentrations determined by different methods. These are listed in Table 8 for adult subjects (mostly young adults), with some comment regarding the methods used. The mean values obtained range from 1.1 μg. to 12.6 μg. per 100 ml. It seems clear that no uniformly acceptable method for measurement of this steroid has been advanced and that the interpretation of data obtained by any of these methods is subject to certain limitations, some of which have been discussed. Despite these limitations, it appears that, with the modifications presented here, the fluorometric method allows a fairly reliable estimation of corticosterone in small amounts of plasma. The technic is not simple and may not be wholly specific for corticosterone, especially when only small amounts of it are present. The ratio found in this study for plasma concentrations of corticosterone to cortisol was about 3.5 to 1.0. In response to the administration of ACTH in normal subjects there appears to be a relatively small increase in the adrenal secretion of corticosterone, as indicated by a mean elevation in the plasma concentration of only 2.2 μg. per 100 ml.

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STUDY OF THE NORYMBERSKI METHODS FOR DETERMINATION OF 17-KETOGENIC STEROIDS (17-HYDROXYCORTICOSTEROIDS) IN URINE

CHARLES SOBEL, B.S., ORVILLE J. GOLUB, PH.D., RICHARD J. HENRY, M.D., S. L. JACOBS, PH.D. AND GOPAL K. BASU, M.S.

Bio-Science Research Foundation, Los Angeles, California*

ABSTRACT

Modified methods for the determination of 17-ketogenic steroids by direct and indirect procedures are presented. Several factors in the Zimmermann reaction were studied experimentally; namely, time and temperature of color development, composition of reagents, specificity and method for correcting for background absorbance. The stoichiometry of the bismuthate oxidation reaction was studied and shown to effect satisfactory recoveries. Conditions for quantitative reduction of 17-ketosteroids by borohydride are presented. The precision of the methods for 17-ketosteroids and 17-ketogenic steroids was determined. From a study of a limited number of urine samples, both the 17-ketosteroids and the 17-ketogenic steroids appear to be relatively stable at room temperature without added preservative.

THE determination of the urinary C_{21} metabolites (17-hydroxycorticosteroids, 11-oxycorticosteroids, 17-ketogenic steroids) of certain adrenocortical hormones (cortisone, cortisol) as an index of adrenal function has been the subject of many investigations in recent years. This subject has been reviewed by Borth (1), who discusses the methods now in common use and some of the results obtained. The publication by Norymberski, Stubbs and West (2) of their procedure for 17-ketogenic steroids (17-KGS) presents a method of reasonable specificity which can be adapted to routine analysis of multiple samples. The present authors have investigated several aspects of the direct and indirect Norymberski procedures (2, 3), including the Zimmermann reaction used for color development of the 17-ketosteroids (17-KS). A modified procedure for 17-KGS is presented, with data as to its specificity and reproducibility.

METHODS

Reagents and Glassware

1. Ethanol, purified. Absolute ethanol is purified as follows: to a liter of absolute ethanol add 2 Gm. of 2,4-dinitrophenyl-hydrazine hydrochloride and 0.5 ml. of concen-

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* Address: 2231 South Carmelina Avenue, Los Angeles 64.

trated hydrochloric acid. Let stand for approximately forty-eight hours. Distill through a 10-inch Vigreaux column, discarding the first and last 100 ml. Redistill through the same column, again discarding the first and last 100 ml.

2. Ethanol, 70 per cent (V/V). Dilute 700 ml. of purified ethanol to 1 liter with distilled water.

3. Ethylene dichloride. Commercial ethylene dichloride is redistilled from an all-glass distilling apparatus and the fraction distilling between 83° and 84° C. is collected.

4. Potassium hydroxide, A. R. grade, saturated aqueous solution.

5. Potassium hydroxide, ethanolic solution. Add 1 volume of reagent No. 4 to 4 volumes of reagent No. 1 and mix. Centrifuge, and use the supernatant fluid. This reagent is prepared just before use.

6. m-Dinitrobenzene, 1.16 per cent in purified ethanol. The Eastman product is purified as follows: dissolve 30 Gm. of reagent in a minimal volume of ethanol by warming on a steam bath. Cool, add 30 ml. of 20 per cent sodium hydroxide solution and let stand thirty minutes. Then add 3 volumes of distilled water with mixing and let stand fifteen minutes. Filter off the crystalline precipitate on a Büchner funnel. Wash the crystals on the funnel with distilled water and suck dry. Redissolve the crystals in a minimal volume of ethanol. Reprecipitate by adding 3 volumes of water. Filter on a Büchner funnel and wash the crystals with distilled water. Suck as dry as possible. Transfer crystals to an evaporating dish and store in a desiccator over anhydrous calcium chloride, under vacuum, for several days or until dry. Store the final product in an amber bottle.

7. Sodium bismuthate, Merck, reagent grade.

8. Sodium bisulfite, 5 per cent solution, freshly prepared.

9. Potassium borohydride, obtainable from Metal Hydrides, Inc., Beverly, Mass.

10. Dehydroepiandrosterone (DHA) standard. Dissolve 10 mg. of DHA in 100 ml. of purified ethanol. 1 ml. = 100 μg. of DHA.

11. Special glassware: glass-stoppered, heavy-walled centrifuge tubes of 35-ml. and 50-ml. capacity.

Procedures

A. *17-ketosteroids (17-KS)*

The hydrolysis and extraction of these steroids are carried out essentially as described by Drekter *et al.* (4). The details of the method as performed in this study are as follows:

1. Test urine with pH paper. If alkaline, acidify with glacial acetic acid so that any phosphate precipitate which may be present is redissolved.

2. Transfer 8 ml. of urine to a 35-ml. glass-stoppered, pointed centrifuge tube, add 2 ml. glacial acetic acid and 3 ml. concentrated hydrochloric acid

3. Place in boiling water bath for ten minutes and then cool in cold tap water.

4. Add 10 ml. of ethylene dichloride and place in shaking machine for fifteen minutes.

5. Centrifuge, and aspirate off the urine as completely as possible.

6. Add to the solvent 25 to 30 pellets of sodium hydroxide and place in shaking machine for fifteen minutes.

7. Centrifuge, and filter the supernatant solvent through 7-cm. Whatman No. 1 filter paper.

8. Transfer 2.5 ml. of the filtrate (if very low concentrations are expected, use 5.0 ml.) to a 5×1-inch test tube and evaporate to dryness under nitrogen, in a water bath at 50°–55° C. The residue represents 2 ml. of urine.

9. Carry out the Zimmermann reaction on the residue as follows:

(a) To a "blank" tube, the urine residue, and a "standard" tube containing 50 μg. of DHA add 0.2 ml. of m-dinitrobenzene reagent.

(b) Add 0.2 ml. of freshly prepared alcoholic potassium hydroxide reagent and mix.
(c) Place the tubes in a water bath at 25° C. in the dark for thirty minutes.
(d) Add to each tube 5 ml. of 70 per cent ethanol and mix. Measure the absorbances in a Beckman spectrophotometer (model DU or B) at 480, 520 and 560 mμ, using the "blank" as a reference solution.
(e) Calculate "corrected" absorbances of the sample and standard by the following equation:
"Corrected" absorbance $= 2\ A_{520} - (A_{480} + A_{560})$
(f) Calculate 24-hour excretion of 17-KS as follows:

$$\text{mg. 17-ketosteroids per 24 hours} = \frac{\text{corrected sample absorbance}}{\text{corrected standard absorbance}} \times 0.05 \times \frac{\text{24-hr. urine volume (in ml.)}}{2}$$

B. *17-Ketogenic steroids (17-KGS); indirect procedure*

1. The pH of the urine specimen should be determined with pH paper and, if necessary, the urine acidified as described in the 17-KS procedure.

2. Test for reducing sugar with Benedict's reagent. If the result is negative, proceed to Step 3. If positive, one of the following alternative procedures should be employed before proceeding to Step 3.

(a) Yeast fermentation, if the urinary reducing sugar is glucose.
(b) Increasing the amount of sodium bismuthate. This method is satisfactory with low concentrations of sugar.
(c) Extraction of free and conjugated steroids by the method of Edwards, Kellie and Wade (5). This method is superior to either yeast fermentation or increasing the bismuthate. The extraction is carried out as follows: Transfer 20 ml. of urine to a 50-ml. glass-stoppered centrifuge tube, add 10 Gm. of ammonium sulfate and mix to dissolve the salt. Extract three times with 20-ml. portions of solvent (3 volumes of ether plus 1 volume of ethanol). Evaporate the combined extracts to dryness under nitrogen, in a water bath at 50° C. Add 10 ml of ethanol to the residue and warm in hot water to dissolve (with some urines an appreciable amount of insoluble residue may remain). Cool, transfer the solution to a tube and centrifuge. Two aliquots of the supernatant fluid are used. For determination of 17-KS, transfer 4 ml. (equivalent to 8 ml. of urine) to a 35-ml. glass-stoppered centrifuge tube and evaporate off the solvent under nitrogen, in a water bath at 50°–55° C. Dissolve the residue in 2 ml. of glacial acetic acid. Add 8 ml. of water and 3 ml. of concentrated hydrochloric acid. Then determine the 17-ketosteroids, beginning with step 3 of procedure A.

Also transfer a 4-ml. aliquot of the ethanol solution to a 25×100 mm. tube and evaporate off the solvent as described. This residue is used for determination of 17-KGS. Dissolve the residue (equivalent to 8 ml. urine) in 8 ml. of glacial acetic acid and add 8 ml. of distilled water. Proceed to Step 3, beginning with addition of sodium bismuthate.

3. Transfer 8 ml. of urine to a 25×100 mm. tube. Add 8 ml. of glacial acetic acid and 2.0 Gm. of sodium bismuthate. Stopper with a clean rubber stopper.

4. Place in a shaking machine for thirty minutes. Urine samples in contact with bismuthate should be protected from light. The samples may be covered with a heavy black cloth during the treatment with bismuthate.

5. Centrifuge, and decant the supernatant fluid into another centrifuge tube. Add 2.0

Gm. of sodium bismuthate and place in a shaking machine for fifteen minutes. Remove from the machine and let stand overnight at room temperature. The following morning the tube is again placed in the shaking machine for fifteen minutes.

6. Centrifuge, and transfer 6 ml. of the supernatant fluid to a 35-ml. glass-stoppered centrifuge tube containing 1.5 ml. of sodium bisulfite.

7. Let stand five minutes, then add 5 ml. of water and 3.6 ml. of concentrated hydrochloric acid. Let stand fifteen minutes.

8. Place in a boiling water bath for ten minutes. Remove to a cold water bath.

9. Add 12 ml. of ethylene dichloride and place in shaking machine for fifteen minutes. Centrifuge.

10. Aspirate off the upper aqueous phase as completely as possible. Avoid excessive loss of solvent phase.

11. Add to the solvent phase 25-30 pellets of sodium hydroxide. Place in shaking machine for fifteen minutes. Centrifuge, and filter through 7-cm. Whatman No. 1 filter paper.

12. Transfer 4 ml. of filtrate (equivalent to 1 ml. of urine) to a 5×1/2 inch test tube and evaporate to dryness under nitrogen, in a water bath at 50°–55° C. In the case of very dilute urines, use 8 ml. of filtrate (equivalent to 2 ml. of urine).

13. Carry out Zimmermann reaction and measure absorbance as described in method for 17-KS.

14. Calculations.

mg. total 17-ketogenic steroids per 24 hrs.

$$= \frac{\text{corrected sample absorbance}}{\text{corrected standard absorbance}} \times 0.05 \times \text{total volume of urine (in ml.)}$$

"Total" 17-ketogenic steroids = sum of the preformed 17-ketosteroids and the 17-ketosteroids resulting from bismuthate oxidation of C_{21} steroids.

"17-Ketogenic steroids" = total 17-ketogenic steroids minus 17-ketosteroids.

C. *17-Ketogenic steroids (17-KGS); direct procedure*

1. Transfer to a 125-ml. Erlenmeyer flask, 8 ml. of urine prepared as in steps 1 and 2 of the indirect procedure, except that, when reducing sugar is present and an ether-alcohol extraction of the steroids has been made, the final residue is dissolved in 0.5 ml. of alcohol (instead of acetic acid) and diluted to 8 ml. with water. Add 100 mg. of potassium borohydride. Check pH. If not over 8, add an additional 25 mg. of borohydride. Let stand one hour at room temperature.

2. Add 8 ml. of glacial acetic acid and let stand forty-five minutes to decompose excess borohydride.

3. Transfer to a 25×100 mm. test tube. Add 2.0 Gm. of sodium bismuthate and proceed as for indirect method, beginning with Step 4.

Calculation:

mg. 17-ketogenic steroids per 24 hrs.

$$= \frac{\text{corrected sample absorbance}}{\text{corrected standard absorbance}} \times 0.05 \times \text{total volume of urine (in ml.)}$$

EXPERIMENTAL

All steroids used were from the U.S.P. Reference collection, except the DHA, which was obtained from General Biochemicals, Chagrin Falls, Ohio.

I. *Zimmermann Reaction*

Earlier studies on this reaction have been reviewed by Munson and Kenny (6). The present authors investigated the following factors:

(a) *Alcoholic potassium-hydroxide reagent.* Two different alkaline reagents have been in common use, namely, aqueous and alcoholic KOH. The former yields colors of much less intensity than the alcoholic reagent and the latter has the disadvantage of being unstable. Various procedures have been proposed for stabilizing the alcoholic KOH (7, 8) which, in the experience of the present authors, have not achieved complete success. The method given in this paper (see Reagents, No. 5) provides a reagent which is simple to prepare daily, avoids the problem of instability, and yields a very low uncorrected blank absorbance (about 0.03 at 520 mμ in a 5-ml. volume).

TABLE 1. EFFECT OF TIME ON COLOR DEVELOPMENT AT 25° C.

	A_{520}* (corrected)			
	15 min.	30 min.	45 min.	60 min.
Urine sample	0.155	0.197	0.203	0.195
DHA standard (50 μg.)	0.172	0.199	0.208	0.209
Sample/Standard	0.902	0.988	0.976	0.934

* Absorbance at 520 mμ.

(b) *Time required for color development.* The following periods of color development were compared at 25° C., using a urine sample and a DHA standard: fifteen, thirty, forty-five and sixty minutes. The results are presented in Table 1. It is seen that, within experimental error, there was no significant difference in the results obtained between thirty and sixty minutes. A 30-minute period for color development, therefore, was considered adequate and utilized in the standard procedure.

(c) *Temperature for color development.* A comparison was made of colors developed at three temperatures, 25°, 30° and 37° C., using both a urine sample and a DHA standard with both thirty and forty-five minutes for color development. The results are shown in Table 2. From the data obtained it is evident that temperature differences over the range of 25° to 37° C. do not markedly affect the absorbances obtained, although there may be a slight decrease in the ratio of sample absorbance to standard absorbance when the color is developed at 37° C. for forty-five minutes. A temperature of 25° C. was selected for color development.

(d) *Diluting fluid.* A 50-μg. sample of DHA was treated with 0.2 ml. of m-dinitrobenzene reagent and 0.2 ml. of ethanolic potassium hydroxide

TABLE 2. EFFECT OF TEMPERATURE ON COLOR DEVELOPMENT

	A_{520} (corrected)					
	30 minutes			45 minutes		
	25°	30°	37°	25°	30°	37°
Urine Sample	0.171	0.189	0.180	0.184	0.181	0.150
DHA standard (50 μg.)	0.204	0.225	0.216	0.222	0.219	0.192
Sample/Standard	0.838	0.840	0.835	0.830	0.825	0.780

reagent. Color development was carried out in the dark for thirty minutes at 25° C., followed by the addition of 5 ml. of diluting fluid, containing 50, 70 or 100 per cent ethanol. The absorbances were measured immediately and again after standing thirty minutes. The results are listed in Table 3. The diluting fluid containing 70 per cent ethanol, the most commonly used concentration, was retained for the routine procedure.

(e) *Specificity of the Zimmermann reaction.* Marlow (9) reviewed this subject and concluded that maximum color development in the Zimmermann reaction depends upon a carbonyl group being at position 17 and a methylene carbon at 16. He further concluded that no other group on, or modification of, Ring D of the perhydro-1,2-cyclopentanophenanthrene nucleus yields a positive result. Wilson (10) has applied the Zimmermann reaction to a large number of C_{19} and C_{21} ketosteroids. Only 17-ketosteroids give rise to the typical purple color with a maximal absorbance at about 520 mμ. However, with the Zimmermann reagents, all ketosteroids produce some color with appreciable absorption at 520 mμ. In Table 4 are listed the absorbances of a number of ketosteroids subjected to the Zimmermann reaction. The absorbances are "corrected" by the base-line technique as described. It is apparent that, with this technique, ketosteroids lacking a ketone group at C_{17} do not interfere significantly with the procedure for determining 17-KS.

The possible interference by nonketonic substances present in urine was

TABLE 3. EFFECT OF ETHANOL CONTENT OF THE DILUTING FLUID ON COLOR DEVELOPMENT

% Ethanol (V/V) in diluting fluid	A_{520} (corrected)	
	Read immediately	Read after 30 mins.
100	0.235	0.217
70	0.220	0.208
50	0.189	0.174

TABLE 4. CHROMOGENIC VALUES OF VARIOUS KETOSTEROIDS. DATA GIVEN ARE "CORRECTED" ABSORBANCES IN PER CENT OF ABSORBANCE GIVEN BY AN EQUAL WEIGHT OF ANDROSTERONE

Steroids (S)	$\frac{A_s}{\text{Androsterone}} \times 100$
Δ^4-Androstene-3, 17-dione	104
3α-Hydroxyandrostane-17-one (androsterone)	100
Etiocholane-3, 11, 17-trione	145
Δ^4-Androstene-3, 11, 17-trione (andrenosterone)	124
3α-Hydroxyetiocholane-11, 17-dione	185
3β, 11β-Dihydroxyandrostane-17-one	74
3α, 11β-Dihydroxyetiocholane-17-one	100
3β-Hydroxy-Δ^5-androstene-17-one (dehydroepiandrosterone)	144
11β, 21-Dihydroxy-Δ^4-pregnene-3, 20-dione (corticosterone)	0
17α, 21-Dihydroxy-Δ^4-pregnene-3, 20-dione (compound S)	2
17α-Hydroxy-Δ^4-pregnene-3, 20-dione (17-hydroxyprogesterone)	10
17α, 21-Dihydroxypregnane-3, 20-dione	3
3α, 17α, 21-Trihydroxypregnane-20-one (Tetrahydro S)	0
21-Hydroxy-Δ^4-pregnene-3, 20-dione (Desoxycorticosterone)	0
11β, 17α, 21-Trihydroxypregnane-3, 20-dione	0
21-Hydroxy-Δ^4-pregnene-3, 11, 20-trione (compound A)	0
17α, 21-Dihydroxypregnane-3, 11, 20-trione	6
17α, 21-Dihydroxy-Δ^4-pregnene-3, 11, 20-trione (Compound E)	3
11β, 17α, 21-Trihydroxy-Δ^4-pregnene-3, 20-dione (Compound F)	0
3α, 17α, 21-Trihydroxypregnane-11, 20-dione (Tetrahydro E)	8
3α, 11β, 17α, 21-Tetrahydroxypregnane-20-one (Tetrahydro F)	0

also studied. 17-Ketosteroids were determined in several urine specimens both before and after treatment with borohydride (3), which transforms 17-ketosteroids to alcohols (considered to be nonreactive with the Zimmermann reagent). The results (Table 7) indicate that, at least in the urine specimens tested, there was no serious interference from nonketonic substances.

II. *Determination of 17-Ketosteroids (17-KS)*

(a) *Correction for background absorbance.* The three wavelengths used for the "base-line" technique (11) were selected after plotting absorbance curves for urine specimens containing negligibly small concentrations of 17-KS, for normal urine specimens subjected to borohydride treatment, and for DHA standards. Figure 1 shows the absorbance curves for 5 urine specimens with a very low content of 17-KS. Curves 1, 2 and 3 represent data on children's urines, and Curves 4, 5 and 6 on urines from adults with Addison's disease. Curve 7 is that of a DHA standard showing an absorb-

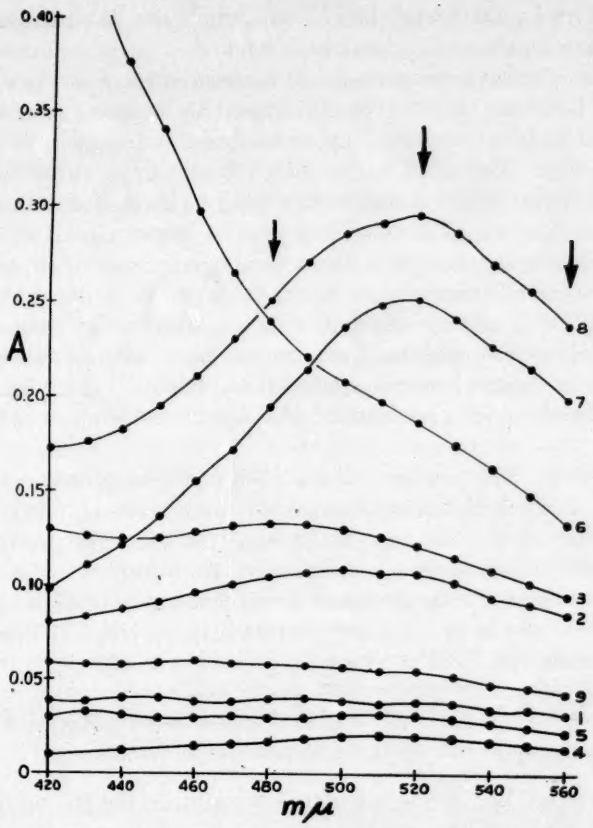

FIG. 1. *Absorption Curves Obtained in the Determination of 17-KS.* Beckman Model B Spectrophotometer. Curve 1—child, 0.24 mg. 17-KS/liter. Curve 2—child, 1.25 mg. 17-KS/liter. Curve 3—child, 1.08 mg. 17-KS/liter. Curve 4—adult, 0.7 mg. 17-KS/liter. Curve 5—adult, 0.9 mg. 17-KS/liter. Curve 6—adult, 0.4 mg. KS/liter. Curve 7—12.5 mg. DHA/liter. Curve 8—adult, 14.6 mg. 17-KS/liter. Curve 9—same urine as in Curve 8 after borohydride reduction. Arrows indicate the 3 wavelengths used in the base-line technique.

ance peak at 520 mμ. Curve 8 represents data on an adult subject's urine with an absorbance peak at 520 mμ; Curve 9 shows the values for the same urine after reduction of the 17-ketosteroids present to the corresponding alcohols by borohydride treatment (*cf.* direct method for determination of 17-KGS). It is seen that Curves 1 to 6 are practically straight lines in the region 480–560 mμ; furthermore, the curve obtained after destruction of 17-ketosteroids by borohydride is also straight in the same region (Curve

9). The curves for DHA and the 17-ketosteroids in a urine extract (Curves 7 and 8) show an absorption peak at 520 mμ.

(b) *Effect of urine extracts on the Zimmermann reaction.* As reported by Sloan and Lowrey (12), the color produced by a given amount of DHA when added to urine extracts may be appreciably less than that obtained in pure solution. The effect varies with different urine specimens and, for greatest accuracy, internal standards should be used. For ordinary clinical applications, a mean correction factor can be employed. In a study of 100 urine specimens, the present authors found that the color suppression due to the presence of urine extract may amount to 0–21 per cent of the absorbance of DHA in pure solution, with a mean of about 10 per cent. For routine analysis of urines we therefore calculate with allowance for a 10 per cent suppression of the color by utilizing the average internal standard value in the formulae given under *Methods* for calculation of 17-KS and 17-KGS.

(c) *Precision.* The precision of the 17-KS procedure was determined at two levels. Eight replicate determinations were made on a urine specimen for which the mean value was low—4.8 mg. per liter. The precision (95 per cent confidence limits) was ±8.1 per cent. Eight replicate determinations were performed on a urine specimen in which the level of 17-KS was higher. The mean value was 13.1 mg. per liter and the precision (95 per cent confidence limits) was ±3.2 per cent.

III. *Determination of 17-Ketogenic Steroids (17-KGS)*

A. *Indirect method*

The value for 17-KGS is calculated by subtracting the amount of preformed 17-KS from the amount of total 17-KS found after bismuthate oxidation.

(a) *Time required for oxidation.* Early in this study of Norymberski's procedure it was found that some urine specimens gave low yields of 17-KGS when bismuthate oxidation was carried out for thirty minutes as recommended by Norymberski, Stubbs and West (2). By extending the oxidation procedure to about sixteen hours (overnight) as recommended by Diczfalusy *et al.* (13), and doubling the amount of sodium bismuthate, maximal yields were obtained in these cases. With some other urine specimens, increasing the oxidation time did not increase the yield of 17-KGS. In the routine method, overnight oxidation is used in order to assure maximal yield from all urine samples.

(b) *Interference by reducing sugars.* The observation of Norymberski *et al.* (2) that the presence of reducing sugars in urine interferes seriously with bismuthate oxidation was confirmed. All urine specimens should be

routinely tested with Benedict's qualitative reagent. If appreciable amounts of reducing sugars are shown to be present by Benedict's test, the urinary steroids (both free and conjugated) are first extracted by the procedure of Edwards, Kellie and Wade (5); the extract thus obtained is redissolved in acetic acid and water and further treated as described in the routine method for 17-KGS.

It has been pointed out (3) that reduction with borohydride will convert 17-20,ketols, unsubstituted at C_{21}, to 17,20,diols (Type 3) which are measured as 17-ketogenic steroids. The indirect procedure, without borohydride, does not determine 17-20,ketols, unsubstituted at C_{21}. These compounds are not normally found in significant amounts, but may appear in certain cases of adrenal dysfunction, and thus there may be instances in which the results of the two procedures will not agree.

(c) *Effect of chlorides.* Smith and Tompsett (14) have reported that, in the presence of chlorides, Zimmermann chromogens are produced from native 17-hydroxyandrostanes during hot, acid hydrolysis of urine samples; and, furthermore, some destruction of chromogens occurs as a result of the hydrolytic procedure. Norymberski and Stubbs (15) have shown that the urea in urine is effective in inhibiting these reactions. The present authors have obtained some evidence which confirms the work of Norymberski and Stubbs. 17-KGS were determined in 1 urine specimen before and after addition of sodium chloride (to a concentration of 2.4 per cent); no difference was found. From the results reported by Norymberski and Stubbs, it would appear advantageous to add urea to urine specimens low in urea (*e.g.*, specimens which have become alkaline due to conversion of urea to ammonia).

(d) *Stoichiometry of the bismuthate oxidation reaction.* Norymberski's 17-KGS method depends on the formation of C_{19},17-ketosteroids by bismuthate oxidation of C_{21} steroids with side-chains of the following types:

1) 17,20,21-triol
2) 17,21-diol,20-one
3) 17,20-diol,21-methyl

C_{21} compounds of these structures include cortisone, cortisol, compound S, and the tetrahydro derivatives of these 3 compounds, as well as pregnanetriol, 11-hydroxy- and 11-ketopregnanetriol. All of these steroids have been isolated from urine. The yield of 17-KS obtained by bismuthate oxidation of some of these steroids is shown in Table 5. In the procedure employed for testing each of the C_{21} steroids, the following samples were oxidized by bismuthate and the total 17-KS determined:

1. Urine (A_1)
2. Same urine plus 30 μg. of the C_{21} steroid (A_2)

TABLE 5. BISMUTHATE OXIDATION OF C_{21} STEROIDS TO C_{19}, 17-KETOSTEROIDS

C_{21} steroid (30 μg.)	17-KS formed by bismuthate oxidation	Yield	
		Theoretical (μg.)	Found (% of theoretical)
Cortisone	adrenosterone	24.9	87
Tetrahydrocortisone	3α-hydroxyetiocholane-11,17-dione	24.9	98
Tetrahydrocortiso'	3α,11β-dihydroxyetiocholane-17-one	24.9	92
Compound S	Δ^4-androstene-3, 17-dione	24.6	97
Tetrahydro S	3α-hydroxyetiocholane-17-one	24.9	98

3. Same urine plus equivalent amount of the specific 17-KS which is presumed (16) to be obtained by bismuthate oxidation of the C_{21} steroid used (A_3)

$A_2 - A_1$ = absorbance of the 17-KS produced by bismuthate oxidation of the added C_{21} steroid.

$A_3 - A_1$ = absorbance of the added 17-KS.

$\frac{A_2 - A_1}{A_3 - A_1} \times 100$ = yield of 17-KS in per cent of theoretical amount.

(e) *Precision.* The precision of the 17-KGS determination was determined at two levels. In 8 replicate determinations on 1 urine the mean levels were 4.8 mg. and 4.0 mg. per liter for 17-KS and 17-KGS, respectively. The precision (95 per cent confidence limits) for 17-KGS was ±16.8 per cent. Eight replicate determinations on another urine specimen whose mean 17-KS and mean 17-KGS were 13.1 and 16.3 mg. per liter, respectively, had a precision of ±13.2 per cent for the 17-KGS.

B. *Direct method*

A direct method for the determination of 17-KGS is desirable since it would not involve a separate determination of 17-KS and is inherently more precise than an indirect method wherein the value is calculated by difference. Appleby *et al.* (3) have presented such a direct method, in which the preformed 17-KS are removed by borohydride reduction to the corresponding alcohols. The 17-KGS are then oxidized by bismuthate and the resulting 17-KS are determined. These then represent a quantitative measurement of the C_{21} steroids from which they were derived.

(a) *Time required for borohydride reduction.* In the original procedure as described by Appleby *et al.* (3), overnight reduction with borohydride was used. As seen from Table 6, a reduction time of one hour is sufficient.

(b) *Efficiency of the borohydride reduction of 17-KS.* A series of urine samples was treated as follows: 1) a 17-ketosteroid determination was per-

TABLE 6. EFFECT OF VARIATION IN TIME OF BOROHYDRIDE REDUCTION ON 17-KGS DETERMINATION

Urine sample	17-KGS (mg./24 hrs.)		
	1 hr. reduction	2 hrs. reduction	Overnight reduction
1	15.2	14.1	15.8
2	22.5	23.5	23.2
3	44.2	44.5	39.0
4	63.2	63.8	60.0
5	82.5	81.0	75.0

formed on each sample before and after borohydride treatment (columns 2 and 3, Table 7); 2) a 17-ketogenic steroid determination was made on each sample by the indirect method (column 4), and 3) an aliquot of each urine sample was oxidized with bismuthate, the total 17-ketosteroids extracted, reduced by borohydride, and Zimmermann chromogens determined on the extracts (column 5). It is evident from Table 7 that the borohydride treatment removes all but a small percentage of the 17-KS.

(c) *Comparison of 17-KGS determined by direct and indirect methods.* Table 8 lists the comparative results obtained on 35 random urine samples. In all but the last 2 instances, there was good correlation. These 2 exceptions were rechecked several times and the cause for the discrepancy is unknown.

(d) *Precision.* In 8 replicate determinations on 1 urine by the direct method, the mean value was 16.8 mg. per liter, with 95 per cent confidence limits of ± 9.5 per cent.

TABLE 7. EFFICIENCY OF THE BOROHYDRIDE REDUCTION OF 17-KETOSTEROIDS

Urine sample	17-KS (mg./24 hrs.)		17-KGS (mg./24 hrs.)	
	Untreated	After borohydride	Untreated	After borohydride
1	6.4	0.7	12.5	0.2
2	5.6	0.6	11.8	0.5
3	19.6	0.1	25.4	0.5
4	13.1	0.9	16.2	1.4
5	5.8	0.8	5.0	0.8
6	—	—	88.7	1.0
7	—	—	56.7	1.6
8	24.3	1.7	—	—
9	80.5	5.5	—	—

TABLE 8. COMPARISON OF DIRECT WITH INDIRECT 17-KGS DETERMINATIONS (MG./24 HRS.)

Direct	Indirect	Direct	Indirect	Direct	Indirect
22.9	21.0	8.0	6.9	1.3	1.9
83.0	75.7	5.0	4.8	26.7	26.4
14.2	13.8	65.8	60.8	6.9	7.3
40.0	34.4	8.3	9.1	5.8	3.9
24.8	22.3	21.8	22.9	11.4	9.6
63.2	56.7	5.5	4.7	3.4	3.7
82.5	88.7	9.7	9.9	14.4	13.4
16.6	15.2	11.0	10.4	16.1	15.9
1.1	1.0	1.6	0.3	20.2	15.5
0.0	0.0	12.7	13.2	11.1	8.7
4.1	7.3	132.0	123.0	79.8	46.7
		4.3	5.0	13.7	43.0

IV. *Stability of Urine Samples*

Seven urine samples with 17-KS and 17-KGS contents ranging from low to high levels, were analyzed when fresh and also after standing one week at room temperature without a preservative. No significant change in levels was observed.

DISCUSSION

The 17-KGS procedures herein described are admirably suited to analysis of multiple samples without requiring specialized equipment. In contrast to those procedures utilizing the Porter-Silber reaction, which determines only steroids possessing the 17,21-diol-20-one side-chain (2), the 17-KGS procedures include in their determination such important urinary steroids as pregnanetriol and tetrahydro S. This would appear to be a distinct advantage for clinical diagnosis. The precision of these 17-KGS methods is considered to be satisfactory for clinical purposes.

The use of the "base-line" technique for correction of background absorbance is obviously based on the assumption that the absorbances of nonspecific materials at the three wave-lengths chosen lie on a straight line. Some evidence is presented to strengthen this assumption, but there may be urines which do not fulfill this requirement. Additional support is furnished, however, by the consistent finding with this procedure of insignificant amounts of steroids in the urine of infants and Addisonians and the fact that treatment of the urine of adult subjects with borohydride causes a transformation from a peak absorbance to a straight-line curve.

Normal values for urinary 17-KGS in adults have been adopted in our laboratories as follows: 8–25 mg. per twenty-four hours for males and 5–18

mg. per twenty-four hours for females. These values are based on those previously reported for this method (2, 13, 17, 18).

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Letters to the Editor

STUDIES ON THE ORIGIN OF ALDOSTERONE DURING HUMAN PREGNANCY*

To the Editor:

A progressive increase in urinary excretion of aldosterone occurs normally during human pregnancy (1). In this medical center (2), levels reached during the third trimester (salt intake, 7–8 Gm.) have ranged from 21 to 125 μg. per day, with a mean of 47 μg.; in non-pregnant women the levels are less than 10 μg. per day. The increased urinary aldosterone during pregnancy may represent increased secretion of the hormone from the maternal adrenal cortex, or possibly production from some extra-adrenal source such as the placenta or fetal adrenal. An opportunity arose to investigate these alternatives with the occurrence of pregnancy in 2 women who had previously undergone complete bilateral adrenalectomy for Cushing's syndrome.

CASE REPORTS AND METHODS

Mrs. E.K.

This 40-year-old patient was admitted to the Toronto General Hospital in June 1955, because of shortness of breath and signs of cardiac failure. She had hypertension (220/140), some loss of scalp hair and truncal obesity. She had had diabetes mellitus for five years and amenorrhea for one year. The clinical diagnosis of Cushing's syndrome was confirmed by the finding of high urinary 17-hydroxycorticoids (resting state) and a hyperactive response to intravenous ACTH. On June 27, 1955, total bilateral adrenalectomy was performed. Pathological report—bilateral hyperplasia; total weight of adrenals, 32 Gm. The immediate postoperative course was uneventful and stabilization was reached during therapy with 50 mg. of hydrocortisone (F) daily. She gradually lost the stigmata of Cushing's syndrome, but hypertension persisted. Within several months, postural hypotension (pressure as low as 60/40 when she stood up, in spite of a blood pressure of 200/100 when lying down) became a problem which was eventually controlled by the use of 0.05 mg. of 9-alpha-fluorohydrocortisone (9α-FF) daily.

In November 1955 she became pregnant (last menstrual period November 8; expected date of confinement August 18, 1956). She had had 1 uneventful pregnancy seventeen years previously. She was maintained on 40–50 mg. of F daily and 0.05–0.2 mg. of 9α-FF. The pregnancy was complicated by several factors: 1) difficulty in controlling the blood pressure, as decreasing the dose of 9α-FF to levels required to lower supine blood pressure (which averaged 200/100) caused severe postural hypotension, 2) asymptomatic lues, which was detected and treated by 8 million units of penicillin during the fifth

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month of pregnancy (April 1956), and 3) recurrent urinary-tract infections (usually mixed coliform organisms), with occasional slight elevation of blood nonprotein nitrogen concentration in the latter part of pregnancy.

Although normal fetal heart sounds were present up to the thirty-sixth week, at this time the patient noted that fetal movements had ceased. A cesarean section was performed and a stilborn male child (weight 4 pounds 8 ounces) was delivered.

Mrs. N.B.

This 31-year-old woman was admitted to the Toronto General Hospital in October 1955, with a history of fatigue, easy bruisability, acne, hirsutism, pain in the back and hips, edema of the hands and feet, and amenorrhea of ten months' duration. Her earliest symptoms began five years previously. The clinical diagnosis of Cushing's syndrome was confirmed by high levels of urinary 17-ketosteroids and 17-hydroxycorticoids and a hyperactive response to intravenous ACTH. On November 9, 1955, a bilateral adrenalectomy was performed. Pathological report—bilateral hyperplasia; total weight of adrenals, 20 Gm. The postoperative course was satisfactory, and the stigmata of Cushing's syndrome gradually disappeared. Therapeutic stabilization was finally reached with 25 mg. of cortisone (E) and 0.2 mg. of 9 α-FF daily.

Menses returned on January 16, 1956, and there were two normal periods in February and March respectively. In April 1956 she became pregnant (last menstrual period March 22; expected date of confinement December 29, 1956). She had had 4 previous pregnancies. Throughout her pregnancy, she was maintained on 25 mg. of E and 0.2 mg. of 9α-FF. She gained 18 pounds. Her blood pressure remained at 110/60 and she had minimal transient swelling of her ankles in the last month of pregnancy. On January 5, 1957, a healthy female infant weighing 6 pounds 1 ounce was delivered following a five-hour labor.

Methods

During the periods of study, urine was collected on ice as 24-hour specimens while the patients were being maintained on oral F and 9α-FF, and a diet containing 8 Gm. of salt. Urinary creatinine was measured to check completeness of collections. Daily urinary

TABLE 1. URINARY ALDOSTERONE IN 2 PREGNANT BILATERALLY ADRENALECTOMIZED WOMEN

Patient	Date	Week of pregnancy	NaCl intake (Gm.)	Therapy		Aldosterone in urine (μg./24 hrs.)
				F*	9α-FF*	
Mrs. E.K.	Mar./56	19	8	40	0.2	(i) 1.0 (ii) 1.6
	June/56	32	8	40	0.2	—†
Mrs. N.B.	Dec./56	38	8	20	0.2	4.4
	Jan./57	Post partum	8	20	0.2	<0.5‡

* F = hydrocortisone. 9α-FF = 9α-fluorohydrocortisone.

† No quantitative result, due to accidental loss in last stages of chromatography. No visible aldosterone spot in previous chromatogram.

‡ No aldosterone could be detected.

sodium excretion was determined by means of a flame photometer. The values averaged 140 mEq. per day, indicating that there was approximate sodium balance. Urinary aldosterone determinations were carried out on pooled four-day specimens, according to the microchemical procedure of Gornall *et al.* (3).

Six-liter volumes of urine were extracted at pH 2 with methylene chloride for forty-eight hours. The extract was purified by an alkali wash, a benzene-water transfer, and partition between 50 per cent ethanol and petroleum (boiling range, 60–80). The material was chromatographed successively in the following systems: (a) benzene: 5 per cent ethyl acetate/methanol:water (Bush), (b) toluene/propylene glycol (Zaffaroni), (c) benzene:10 per cent ethyl acetate/methanol:water (Bush), and (d) isooctane/tertiary butanol:water (Eberlein-Bongiovanni). These steps have been found adequate to separate aldosterone in a high state of purity from most urinary extracts. Aldosterone was measured spectrophotometrically by ultraviolent absorption and checked when possible by the dinitrophenylhydrazine and blue-tetrazolium reactions. Results are recorded in Table 1. We have little reason to doubt that the material isolated was aldosterone, though the quantities obtained did not permit absolute identification.

COMMENTS

In Patient N.B. the finding of a urinary excretion of 4.4 μg. of "aldosterone" per day during the third trimester and less than 0.5 μg. per day during the postpartum period suggests that the placenta or the fetal adrenal cortex, or both, may be a source of small amounts of aldosterone during pregnancy. There is a possibility that traces of aldosterone arose from metabolism of the administered hydrocortisone in these tissues or elsewhere in the body. Whatever the source of the "aldosterone" in these adrenalectomized patients, the amount was *no more than one-tenth* of the average excretion by normal pregnant women in the third trimester. Certainly the major source of urinary aldosterone during pregnancy must be sought outside the placenta or the fetal adrenal. It is probable that the increase is due mainly to increased secretion from the maternal adrenal cortex. Since present methods measure only aldosterone and none of its possible metabolites, part of the increase may be due to a depressed metabolic transformation during pregnancy, as has been demonstrated for hydrocortisone (4, 5). In any case it seems reasonable to conclude that the high output of aldosterone during pregnancy is not a requisite for healthy gestation and serves no apparent purpose.

JOHN C. LAIDLAW, M.D., PH.D.
MAY COHEN, M.D.†
ALLAN G. GORNALL, PH.D.
Departments of Medicine and of Pathological Chemistry,
University of Toronto,
Toronto, Canada

† Medical Research Fellow, National Research Council of Canada.

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SHORT-TERM DECREASE OF SERUM PROTEIN-BOUND IODINE CONCENTRATION AFTER ADMINISTRATION OF 2,4-DINITROPHENOL IN MAN

TO THE EDITOR:

Studies of the physiology of the thyroid have been directed to the regulation of thyroid hormone secretion and to the rôle and fate of this hormone in peripheral oxidative processes. Several recent papers have dealt with the influence of work (1, 2), cold (3, 4) and fever (5, 6). To enhance oxidative processes experimentally, use may be made of 2,4-dinitrophenol. Goldberg and Chaikoff (7–9) have shown that administration of this substance leads to a striking decline in the concentration of protein-bound iodine (PBI) in the serum of rats, without any simultaneous increase in thyrotropic activity. They raise the question whether the TSH-secretion was not inhibited directly by the increased metabolism. Should this be so, then the serum concentration of hormone would not be the only factor regulating the production and secretion of TSH. The most recent observations in man confirming these results are those of Castor and Beierwaltes (10).

Castor and Beierwaltes gave 2,4-dinitrophenol in repeated doses over a period of several days. The interval between the individual PBI estimations was also several days, and was never shorter than one day. In experiments on 2 volunteers, we have found that the decline in PBI concentration can be detected as early as four hours following the administration of a single dose of the drug.

The experiments were performed on 2 healthy medical students for two consecutive days. On the morning of the first day the BMR was measured and the serum PBI level estimated. A dose of 150 mg. of 2,4-dinitrophenol

was administered orally, after which pulse, blood pressure, body temperature and BMR were recorded at hourly intervals. The values of the first three did not change during the course of the experiment, but the BMR increased substantially in about four hours after the administration of the drug. At this time, estimation of the PBI level was repeated. The subjects remained fasting during the whole course of the experiment. The same procedure was repeated on the following day with the exception that the dose of 2,4-dinitrophenol was doubled. This dose of 300 mg. produced a sensation of heat beginning between the second and the third hour. The PBI determinations were made in duplicate according to the method of Barker *et al.* (11). For colorimetry, the modification of Grossmann and Grossmann was used (12).

The results are shown in Table 1. The relatively high initial BMR values

TABLE 1. EFFECT OF DINITROPHENOL ON BMR AND SERUM PROTEIN-BOUND IODINE LEVEL

Subject	Dose of dinitrophenol (mg.)	BMR (%)		PBI (μg./100 ml.)*	
		Initial	4 hrs.	Initial	4 hrs.
G.M.	150	+20	+29	5.4	4.8
	300	+25	+62	4.6	3.1
M.V.	150	+16	+28	2.5	2.0
	300	+30	+60	3.4	2.3

* Means of duplicates.

found in both cases are accounted for by the fact that the estimation was carried out on ambulant subjects. We have no satisfactory explanation for the relatively low initial PBI value in Subject 2 in the first experiment (normal values in our laboratory for serum PBI concentration range from 3.0 to 8.0 μg. per 100 ml.).

It is evident that both doses of 2,4-dinitrophenol caused an increase in the BMR and a decrease in the serum PBI level as quickly as four hours after administration, and that the larger dose had the greater effect.

ŠTEFAN NÉMETH, M.D.

Endocrinological Institute of the Slovak Academy of Sciences,
Ulica Obrancov Mieru 1 a, Bratislava, Czechoslovakia

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OVOGONIA IN RUDIMENTARY GONADS IN A CASE OF TURNER'S SYNDROME WITH MALE SEX CHROMATIN PATTERN

To the Editor:

The finding of a female sex chromatin pattern in men with Klinefelter's syndrome (1–3) was received with considerable skepticism, particularly after Bunge and Bradbury reported their finding that occasional tubules with spermatogonia could be demonstrated (4a). The doubts at first expressed by Bunge and Bradbury (4a) as to the validity of sexing by chromatin patterns apparently were soon dissipated, for in a subsequent communication (4b) these authors state that, "the finding of female type somatic cells in patients with Klinefelter's syndrome whose gonads show male

type germinal cells requires an explanation for a paradox. . . . A working hypothesis which resolves this paradox can be derived from Jost's (5) demonstration of the effects of total ablation of the embryonic testis." It was only a matter of time before the counterpart would be found, *i.e.*, ovogonia in a female with Turner's syndrome and a male sex chromatin pattern. The purpose of this communication is to place such a case on record.

CASE REPORT

A white female aged 8½ years, of small stature (43½ inches in height) exhibited typical Turner's syndrome. There was marked webbing of the neck, high palate, and low posterior hair line. The vaginal smear was of the normal infantile or prepuberal type. Repeated studies of oral mucosal smears and blood smears showed a male sex chromatin pattern. The bone age was slightly retarded. The 24-hour urinary excretion of 17-ketosteroids was 3.8 mg., of 17-hydroxycorticoids 6.9 mg., and of gonadotropin less than 6.6 m.u. The genitalia were normal for a female child of her age; there was no axillary or pubic hair. Studies to determine the presence or absence of coaretation of the aorta were undertaken, but no evidence of this condition was discovered. Anomalies of the genitourinary system were not demonstrable by intravenous pyelogram.

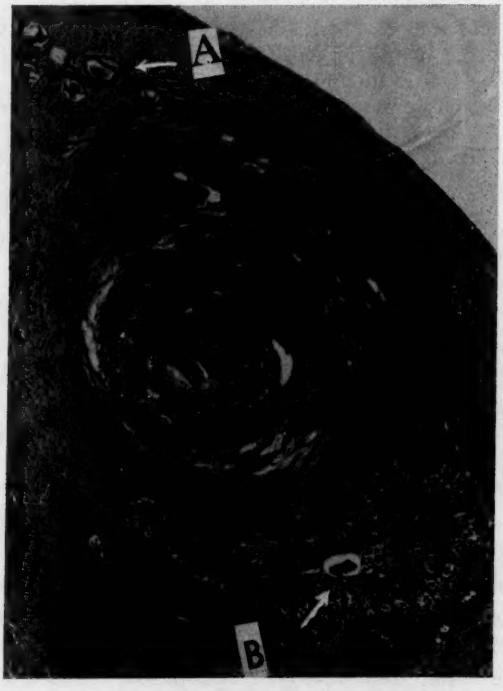

FIG. 1. Note (A) numerous primordial follicles. Arrow points to atretic follicle with captive ovum (B).

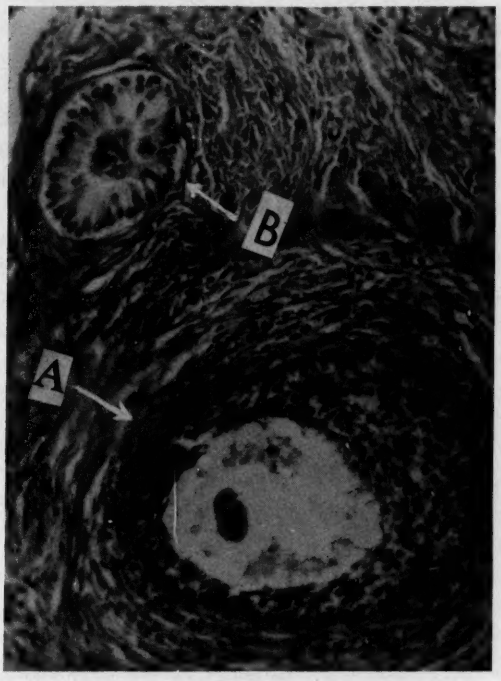

FIG. 2. Note Graafian follicle with degenerating ovum (A) and immature seminiferous tubule (B).

Laparotomy was performed at the University Hospital on July 5, 1957. A rudimentary uterus with elongated Fallopian tubes, and bilateral vestigial gonadal streaks were found. The rudimentary gonadal streak on the right side was slightly cystic (dumb-bell shaped) at its distal end. On the left side just below the gonad there was a minute yellowish nodule in the mesosalpinx, which proved to be an adrenal rest. Histologic study of sections obtained from the rudimentary gonad revealed primordial follicles scattered throughout the cortex (Fig. 1). Histologic study of a section taken through the small cystic dilatation revealed a small follicular cyst. In other sections small atretic follicles were seen, and in the core of one of these a captive ovum was present. One Graafian follicle with a degenerated ovum was noted and occasional seminiferous tubules lined by tall columnar epithelium were observed at the periphery of the cortex (Fig. 2). There was conspicuous absence of Leydig cells in all of the 40 sections examined. The rete ovarii were poorly developed.

This case is of importance in rounding out the spectrum of gonadal arrest and defective development in relationship to hermaphrodism. The presence of ova in a dysgenetic gonad in a girl with a male sex chromatin pattern (Turner's syndrome) is as paradoxical as the finding of spermatogenesis in men with Klinefelter's syndrome and a female sex chromatin pattern.

Turner's syndrome is one of many syndromes which have gonadal dysgenesis as a common factor. Because of the variation in appearance of these patients, several different nomenclatures have been used. However, all have a common fundamental feature, *i.e.*, rudimentary gonadal streaks. These vestigial ridges have been described in the past as "aplastic ovaries," "agenetic ovaries," and "rudimentary ovaries." The histologic structures of these streaks in patients with either a male or female sex chromatin pattern are quite similar. In general the vestigial streak is composed of stromal tissue without primordial follicles. In a few instances primordial follicles and small follicular cysts have been reported. However, in the few cases thus far on record with primordial follicles, the sex chromatin pattern was female (6, 7). In a case very similar to ours which was recently reported by del Castillo and Argonz, sex chromatin studies were not made (8).

The finding of oocytes in the rudimentary gonads of a patient with Turner's syndrome and a male sex chromatin pattern lends further support to the possibility of complete sex reversal in human beings.

ROBERT B. GREENBLATT, M.D.

Department of Endocrinology,
Medical College of Georgia,
Augusta, Georgia

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The Endocrine Society

THE 1958 ANNUAL MEETING

The Fortieth Annual Meeting of The Endocrine Society will be held in the St. Francis Hotel, San Francisco, California, Thursday, Friday and Saturday, *June 19, 20, and 21, 1958.*

The Committee on Local Arrangements—Dr. Roberto Escamilla, Chairman; Dr. Donald E. Bernstein, Dr. Peter H. Forsham, Dr. Minnie B. Goldberg, Dr. Gilbert S. Gordan, Dr. Francis S. Greenspan, Dr. C. H. Li, as members of the committee.

All Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily in the Colonial Ballroom, and in addition there will be simultaneous afternoon sessions in the Italian Room. The annual dinner is scheduled for Friday, June 20 at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All reservations for rooms must be made through the AMA Housing Bureau, Room 300, 61 Grove Street, San Francisco 2, California, after January 1, 1958. No reservations will be accepted before that date. If possible, official application forms as published in the Journal of the American Medical Association should be used. Arrival and departure dates must be stated. All requests must be accompanied by check or money order (not cash) made payable to "The AMA Housing Bureau" in the amount of $10.00 per room, which will be credited to your hotel account. Be sure to state your first, second and third choice of hotels. It is important to make reservations immediately in order to avoid disappointment.

Final program, membership card and advance registration forms will be sent on May 15, 1958 to members whose current dues have been paid.

THE 1958 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

MEDAL OF THE ENDOCRINE SOCIETY

In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. Dr. Carl R. Moore was the recipient in 1955, Dr. Frederick L. Hisaw in 1956, and Dr. Joseph C. Aub in 1957.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl Heller; 1949—Dr. George Sayers; 1950—Dr. Oscar M. Hechter; 1951—Dr. Albert Segaloff; 1952—Dr. Seymour Lieberman; 1953—Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts); 1954—Dr. Isadore M. Rosenberg; 1955—Dr. Jack Gross; 1956—Dr. Alfred M. Bongiovanni; 1957—Dr. Nicholas S. Halmi. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-four months of the date of the award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

THE AYERST AND THE SQUIBB FELLOWSHIPS

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either o ...ese degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

THE SCHERING AND THE UPJOHN SCHOLARS OF THE ENDOCRINE SOCIETY

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The awards will not exceed $2,500 annually for each individual and will be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Medal of the Endocrine Society; the Ciba Award; and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *November 1* each year.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *November 1* each year.

The Awards Committee will meet in November and notice of awards to successful nominees and applicants will be made not later than December 1.

The American Goiter Association

THE 1958 ANNUAL MEETING

The next Annual Meeting of The American Goiter Association will be held in the St. Francis Hotel, San Francisco, California, on *June 17, 18 and 19, 1958.*

All reservations for rooms (accompanied by a $10 deposit) must be made through the AMA Housing Bureau, Room 300, 61 Grove Street, San Francisco 2, California.

TRAINING PROGRAM FOR STEROID BIOCHEMISTRY

Applications are now being accepted for the third course in the Training Program for Steroid Biochemistry which will begin October 1, 1958. The training program is sponsored by the National Cancer Institute of the National Institutes of Health to provide specialized training for persons interested in steroid investigations.

The program is conducted by personnel at the Worcester Foundation for Experimental Biology with the Department of Chemistry, Clark University, Worcester, Massachusetts, and the Department of Biochemistry, College of Medicine, University of Utah, Salt Lake City.

Two groups of candidates will be selected for training. Postdoctoral candidates having an M.D. or a Ph.D. degree will receive $5000 for a one-year training period extending from October 1, 1958 through September 31, 1959. The course will consist of laboratory instruction and lectures covering the theoretical and practical aspects of steroid research, and a period of research on a steroid problem with an established investigator.

Candidates having a B.S. or M.S. degree or equivalent training will receive $1500 for a six months' period extending from October 1, 1958 through March 31, 1959. The pre-doctoral program is intended to provide competency in the analysis of steroid substances for research and clinical laboratories.

The closing date for applications will be *June 1, 1958*. Requests for applications should be made to the Training Program for Steroid Biochemistry, Department of Chemistry, Clark University, Worcester, Massachusetts, or to Dr. Kristen Eik-Nes, Department of Biochemistry, College of Medicine, University of Utah, Salt Lake City, Utah.